Ana Rita Silva Moreira

Development of proliposomes as a vehicle to deliver new molecules with antitumor activity

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Trabalho realizado sob a orientação de:

Professor Doutor Domingos Ferreira

Professora Doutora Madalena Pinto

Doutora Susana Martins

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Author's declaration:

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation. Under the terms of the referred Decree-Law, is hereby declared that the following articles/communications were prepared in the scope of this dissertation.

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* presenting author

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INDEX

INDEX OF FIGURES

INDEX OF TABLES

INDEX OF SCHEMES

ABSTRACT

Xanthone derivatives are frequently isolated from natural sources, having a wide range of pharmacological activities. Thus, these structures have attracted great interest and a large variety of synthetic xanthone derivatives have emerged. **LEM2** is a synthetic xanthone derivative with tested antitumor effect in different cell lines. Xanthone derivatives frequently present poor aqueous solubility and nanosystems might present an attractive strategy to overcome this limitation.

Liposomes represent a versatile system for drug delivery, in the nanometer and micrometer scale. Liposomes have already demonstrated to be adequate systems for the use in cancer chemotherapy. However, liposomes present physical and chemical instability, limiting their shelf-life. In this context, proliposomes, dry phospholipid powders, emerge as an alternative to overcome the instability of liposomes.

In this dissertation, proliposomal formulations were developed to encapsulate the synthetic xanthonic compound **LEM2.** Three methods were used to produce proliposomes: film deposition on carrier, freeze drying and spray drying. After their production, proliposomes were hydrated to form liposomes.

The three methods produced proliposomes which, on hydration, formed liposomes in the nanometer scale, which were efficient in encapsulating **LEM2.** It was found that 30 days after the production of proliposomes by film deposition on carrier and freeze drying, the liposomes obtained by their hydration present altered properties, reflecting some instability. On the contrary, the spray dried proliposomes presented good stability after 30 days of their production, presenting a promising strategy to obtain liposomes with improved stability.

Keywords: xanthone; cancer; antitumor; nanotechnology; liposomes; proliposomes.

RESUMO

Derivados xantónicos são frequentemente isolados de fontes naturais, tendo uma variedade de atividades farmacológicas. Por isso, estas estruturas têm atraído um grande interesse e uma grande variedade de derivados xantónicos sintéticos têm surgido. **LEM2** é um derivado xantónico de origem sintética com efeito antitumoral testado em diferentes linhas celulares. Os derivados xantónicos apresentam frequentemente fraca solubilidade em sistemas aquosos e o uso de nanossistemas poderá representar uma estratégica atrativa para ultrapassar esta limitação.

Os lipossomas representam um sistema versátil para a libertação de fármacos, na escala manométrica e micrométrica. Os lipossomas já demonstraram ser adequados para uso em quimioterapia no cancro. No entanto, os lipossomas apresentam instabilidade física e química, limitando o seu tempo de vida útil. Neste contexto, os prolipossomas, pós de fosfolípidos secos, surgem como uma alternativa para ultrapassar a instabilidade dos lipossomas.

Nesta dissertação, foram desenvolvidas formulações de prolipossomas para encapsular o derivado xantónico sintético **LEM2**. Foram usados três métodos para produzor prolipossomas: *film deposition on carrier***,** liofilização e *spray drying*. Após a sua produção, os prolipossomas foram hidratados e a para formar lipossomas.

Os três métodos produziram prolipossomas que, quando hidratados, conseguiram formar prolipossomas na escala nanométrica, que demonstraram ser eficientes na encapsulação do **LEM2**. Foi descoberto que 30 dias após a produção dos prolipossomas por *film deposition on carrier* and *freeze drying*, os lipossomas gerados pela sua hidratação apresentam propriedades alteradas, refletindo alguma instabilidade. Pelo contrário, os prolipossomas produzidos por *spray drying* apresentaram boa estabilidade ao fim de 30 dias, representando uma estratégia promissora para obter lipossomas com melhor estabilidade.

Palavras-chave: xantona; cancro; antitumoral; nanotecnologia; lipossomas; prolipossomas.

ABBREVIATIONS

ΔH – Enthaplpy variation

BPO - Benzoyl peroxide

CEQUIMED – Centro de Química Medicinal da Universidade do Porto

CH - Cholesterol

DMPC – Dimyristoyl phosphatidylcholine

DPPC – Dipalmitoyl phosphatodylcholine

DSC – Differential scanning calorimetry

EPC - Egg phosphatidylcholine

EPR – Enhanced permeation and retention

FD – Freeze drying

FDC – Film deposition on carrier

HPLC – High Performance Liquid Chromatography

IUPAC - International Union of Pure and Applied Chemistry

LUV – Large unilamellar vesicles

MLV – Multilamellar vesicles

MW – Microwave

NBS - N-bromosuccinimide

PC – Phosphatidylcholine

RES – Reticuloendothelial system

Rf – Retention factor

SD – Spray drying

SPC – Soya phosphatidylcholine

SUV – Small unilamellar vesicles

TLC – Thin layer chromatography

T_m – Phase transition temperature

UV – Ultraviolet light

OUTLINE OF THE DISSERTATION

The present dissertation consists of seven chapters. This dissertation involves three main areas of research: synthesis of formylated xanthone **LEM2,** development of an HPLC method to quantify **LEM2** and development of proliposomal formulations to encapsulate **LEM2.**

CHAPTER 1 – INTRODUCTION

The introductory chapter of the present dissertation is divided in four sections. In the first part, a briefly overview about cancer nanotechnology will be presented and in the second will be focused on liposomes as drug delivery systems suitable for application in cancer nanotechnology, with their advantages and drawbacks being highlighted. In the third part, proliposomes will be presented as a promising strategy to overcome the drawbacks presented by liposomes. And in the fourth part, a brief introduction to xanthone derivatives will be given and their use in nanossystems will be justified.

CHAPTER 2 – AIMS

Herein, the main objectives of the present dissertation are described.

CHAPTER 3 - RESULTS AND DISCUSSION

Results are subdivided in five sections. In the first part, the different reaction steps for the synthesis of carbaldehydic xanthone **LEM2** will be described. The second part will present the development of a HPLC method for the quantification of **LEM2**. The third part will show the analysis of surface morphology and thermal behavior of proliposomes. The fourth part will show the characterization of the liposomes obtained by hydration of proliposomes. The fifth part will present stability results of proliposomes.

CHAPTER 4 – CONCLUSIONS

This chapter includes the general conclusions of the present dissertation.

CHAPTER 5 - MATERIAL AND METHODS

In this chapter, the experimental procedures for the synthesis of **LEM2** will be detailed. The HPLC conditions for the development of an HPLC method to quantify **LEM2** will be detailed. The procedures for the proliposome production and their analysis will be described in detail. The hydration of proliposomes and the characterization of the obtained liposomes will also be described. The conditions used to access the stability of proliposomes will be specified and the statistical tests and software will be identified.

CHAPTER 6 – REFERENCES

The references will be presented at the end of this dissertation. The references followed the American Chemical Society style guide. The main bibliographic research motors were ISI Web of Knowledge, from Thomson Reuters, Scopus, and Google.

CHAPTER 7 – APPENDICES

This section will include the resume of data for the characterization of liposomes obtained by hydration of proliposomes that were used to construct the box and whiskers plots used to present the results.

CHAPTER 1

INTRODUCTION

CHAPTER 1 – INTRODUCTION

1.1. Cancer nanotechnology

Cancer is one of the leading causes of mortality worldwide, with approximately 8.2 million related deaths in 2012 [1]. Cancer begins as localized disease but spreads to different sites within the body, which difficults treatment. The most common treatments are chemotherapy, radiation and surgery. Chemotherapy in general presents a number of drawbacks, such as nonspecific distribution of antitumor drugs, inadequate drug concentrations reaching the tumor site, cytotoxicity, difficult monitoring of therapeutic responses and development of multiple drug resistances [2]. Considering this, there is an emerging necessity to develop alternatives to improve cancer treatment.

Ideally, the therapeutic agent should reach the tumor sites in the desired concentration to destroy the cancerous cells, while minimizing damage to normal cells [2]. Nanotechnology, which commonly refers structures that are up to several nanometers in size, emerges as one of the most promising fields in cancer therapy [3]. Cancer nanotechnology is an upcoming field concerning interdisciplinary research, involving biology, chemistry, engineering and medicine, and its applicability in cancer detection, diagnosis and treatment deserves considerable attention [2, 4].

Nanosystems applied to cancer treatment present unique properties: (i) they can themselves have therapeutic properties; (ii) they might carry a large amount of therapeutic agent; (iii) their surface can be modified with targeting ligands, increasing the affinity and specificity for target cells and tissues; (iv) they can accommodate multiple drug molecules for combinatorial cancer therapy and (v) can overcome drug resistance mechanisms [2].

Nanotechnology can use passive and active targeting strategies to specifically deliver drugs into cancer cells, therefore enhancing the anticancer effect, and simultaneously minimizing toxicity in normal cells. Passive targeting exploits the characteristics of tumor growth (**Figure 1A**), while active targeting is based on molecular recognition processes (**Figure 1B**) [2-3].

Passive targeting takes advantage of the size of nanoparticles and the anatomical and functional differences between normal and tumor vasculature to confine the drug delivery (**Figure 1A**). Commonly, the vasculature of tumors is highly heterogeneous, having areas of

vascular necrosis and densely vascularized areas supplying oxygen and nutrients which enhance tumor growth. Angiogenic blood vessels present a high proportion of proliferating endothelial cells with aberrant underlying basement membrane compared to normal blood cells. In addition, tumor tissues have a leaky and defective architecture, with increased fenestrations between adjacent endothelial cells and the microvessels with enhanced permeability. The tumor lymphatic system is also abnormal, leading to fluid retention in tumors and high interstitial pressure with an outward convective interstitial fluid, which results in metastasis. The combination of the leaky microvasculature and the poor lymphatic drainage results in the enhanced permeation and retention (EPR) effect. This induces the passive targeting of nanocarriers through their accumulation in the tumor at a higher concentration than in the plasma and other tissues, enhancing tumor cytotoxicity (**Figure 1A**) [2, 4].

Active targeting involves the functionalization of surface of nanoparticles with ligands to deliver the drug to the pathological site or to cross biological barriers based on molecular recognition processes (**Figure 1B**). The receptor for the ligand should be expressed exclusively on tumor cells for the recognition to occur in tumor microenvironment. Usually, the internalization occurs via receptor-mediated endocytosis [2, 4].

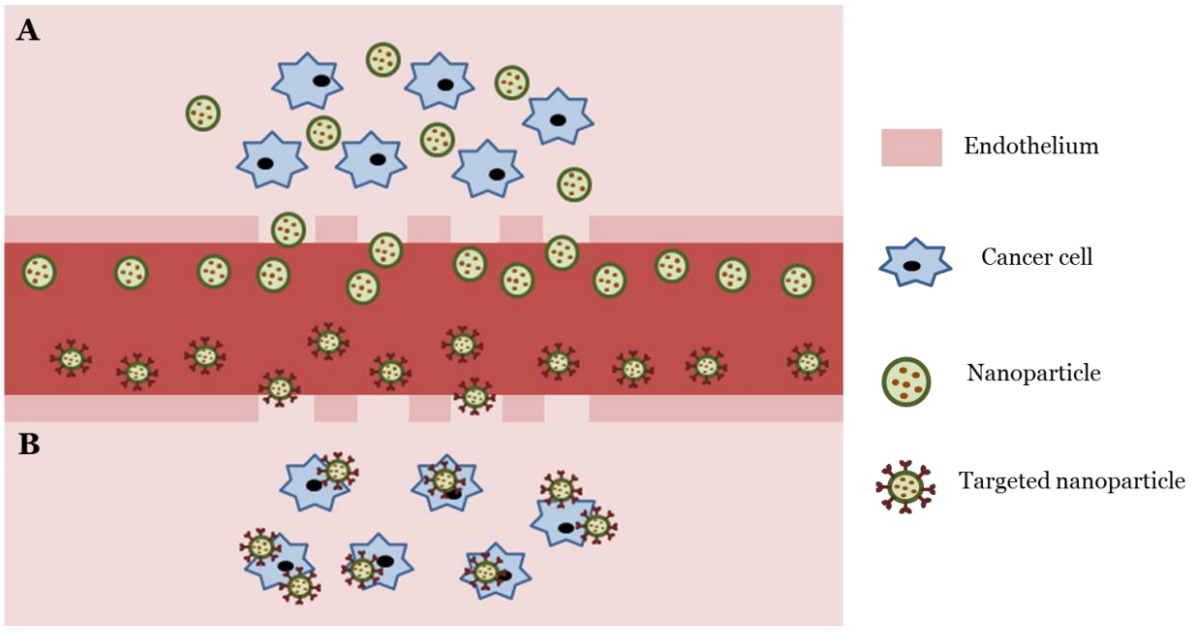


Figure 1 - Schematic representation of passive (**A**) and active (**B**) targeting of nanoparticles to tumors. Figure adapted from [3].

1.2. Liposomes

Liposomes, first reported by Bangham *et al.* in 1965 [5], are microscopic spherical vesicles, in which an aqueous environment is entirely enclosed by a concentric bilayer of phospholipids [5-8]. These vesicular systems are lyotropic liquid crystals with size varying from 20 nanometers to 20 micrometers [9-10]. Liposomes have been extensively studied for their application in drug delivery, drug targeting, controlled release and increased solubility of drugs [7, 10-11].

Liposomes are similar to biological membranes, since they are mainly composed of phospholipids, which are amphiphilic molecules with a hydrophilic head and a hydrophobic tail (**Figure 2**) [10].

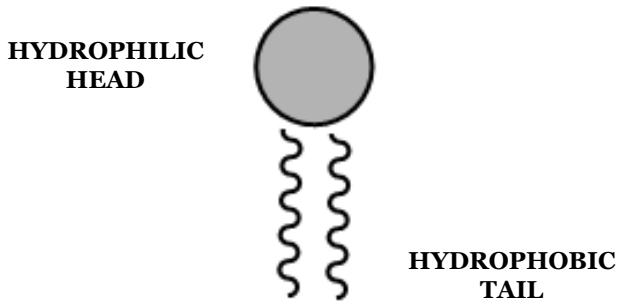


Figure 2 - Representation of the hydrophilic and hydrophobic portions of a phospholipid.

Phospholipids are naturally prone to self-assembly, which leads to their spontaneous aggregation in aqueous environments. In the presence of water, phospholipids align themselves in a thermodynamically stable manner in planar bilayer sheets, minimizing the unfavorable interactions between the bulk aqueous phase and the long fatty acid chains. The heads of the phospholipids form a surface facing the water, while the hydrocarbon tails are repelled by water and face each other, creating a lipid bilayer (**Figure 3A**). Therefore, in a cell, two layers of heads are formed facing the outside and the inside of the cell, attracted to both aqueous environments. The hydrocarbon tails of both layers face each other, thus the resultant structure forms a bilayer (**Figure 3A**). When membrane phospholipids are disrupted, they can reassemble themselves into tiny spheres, smaller than a normal cell,

either as monolayers or bilayers. The monolayer structures are called micelles (**Figure 3B**) and the bilayer structures are liposomes (**Figure 4A**) [8]. The organization of amphiphiles in the form of bilayer sheets occurs due to the high entropy of the system, caused by the interaction forces between the water and the hydrophobic hydrocarbon chains. These interactions are eliminated when the sheets fold themselves into sealed vesicles [8, 10, 12]. Thus, liposomes (**Figure 4**) form spontaneously when the phospholipids are exposed to an aqueous environment, since this is the more energetically stable form [13].

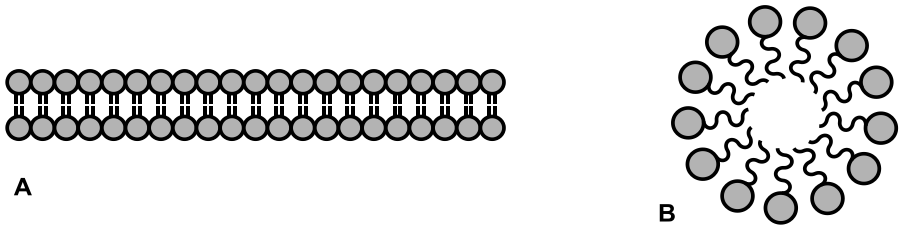


Figure 3 - Schematic representation of monolayer structures formed by phospholipids: lipid bilayer (**A**), micelle (**B**).

Phospholipids confer an amphiphilic nature to liposomes, thus they have a hydrophilic inner core surrounded by a hydrophobic membrane. This vesicular organization allows the entrapment of both hydrophilic and hydrophobic drugs. Water soluble drugs may be solubilized in the internal aqueous compartment of the liposome which difficults and therefore slows down its passage through lipid bilayers. A hydrophobic drug can be dissolved in the hydrophobic part of the liposome or bind to the membrane. Usually, lipophilic drugs exhibit higher encapsulation efficiencies than hydrophilic drugs [8-9]. Lipophilic drugs are more likely to remain encapsulated during storage due to their partition coefficients. They associate with lipid bilayers, thus avoiding leaking out to the exterior water phase [9].

Liposomes can be classified according to the number of bilayers entrapping the internal aqueous volume into unilamellar or multilamellar vesicles. If liposomes have just one bilayer they are classified as unilamellar vesicles (**Figure 4A**), and their properties are similar to those of flat surfaces. Depending on their size, unilamellar vesicles can be separated into small unilamellar vesicles (SUV) with a diameter ranging from 20 to 100 nm, or large unilamellar vesicles (LUV), with a diameter from 100 nm to 1 μm. SUV exhibit large curvature, while LUV presents a low curvature. If there is more than one bilayer, liposomes

are considered to be multilamellar vesicles (MLV) (**Figure 4B**). MLV represent a heterogeneous group with respect to size and morphology of the liposomes. Usually, MLVs present a size range from 100 nm to 20μm. Each concentric layer of liposomes has a thickness of about 4 nm [9-10, 14]. Typically, unilamellar vesicles are suitable for entrapment of hydrophilic drugs in the internal-aqueous space, while MLVs are appropriate to entrap lipophilic drugs [10].

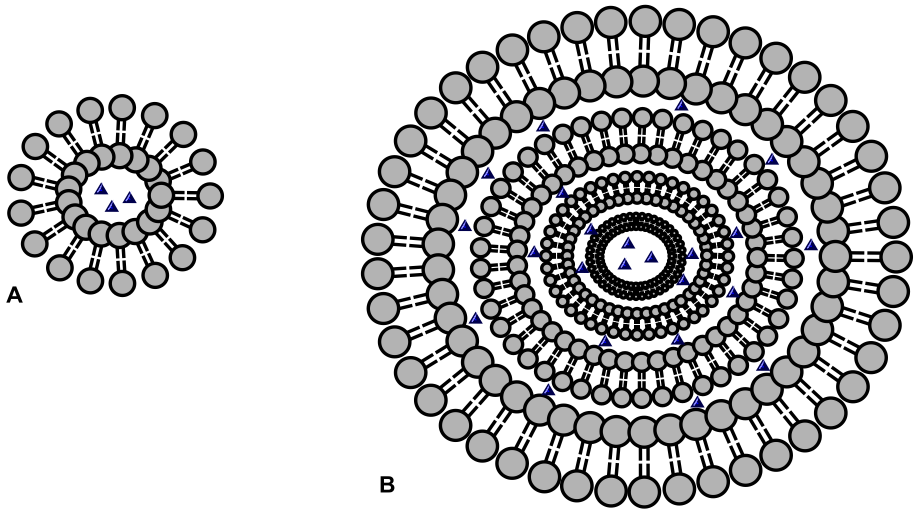


Figure 4 - Schematic representation of two different types of liposomes: unilamellar vesicle (**A**) and multilamellar vesicle (**B**). (▲) represents a water molecule.

Liposomes, as drug delivery systems, need to show appropriate chemical and biological stability. Colloidal stable structures on equilibrium, such as liposomes, are less sensitive to external changes than equilibrium structures, such as micelles. Thereby, liposomes are suitable for pharmaceutical applications. Biological stability is related to the retention of the drug in its target and to the control of the clearance rate of liposomes from the blood system and from certain parts of the body. The clearance rate depends on the dose, size and surface charge of liposomes.

1.2.1. Composition of liposomes

Glycerophospholipids are commonly used phospholipids in liposomal formulations. Glycerophospholipids are composed of a glycerol molecule ($C_3H_8O_3$) covalently attached to two fatty acid chains (with variable levels of saturation) by ester linkages and to a highly polar or charged group by a phosphodiester linkage in the third carbon. Glycerol acts as a backbone, by its attachment to the fatty acid chains and to the phosphate group. The hydrophobic tail of phospholipids is composed by the two fatty acid chains and the hydrophilic head is made of glycerol, phosphate and a polar group [10, 15]. Glycerophospholipids are derivatives of phosphatidic acid (**Figure 5**), in which phosphate group bears a negative charge at neutral pH [15].

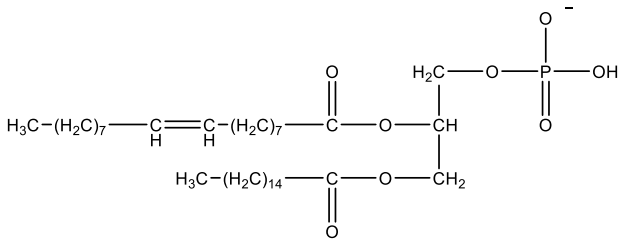


Figure 5 - Phosphatidic acid structure.

The polar head group of glycerophospholipids might be neutral, negatively charged or positively charged [15]. The charge of phospholipids determine the overall surface charge of liposomes [9]. The most common phospholipid is phosphatidylcholine (PC), which has choline as polar head group and presents a neutral net charge (**Figure 6**) [10, 15-16].

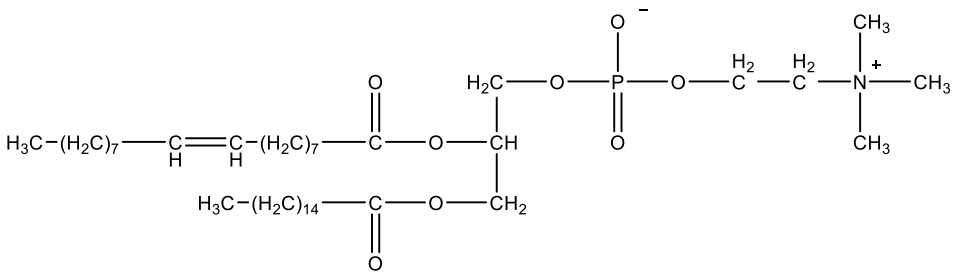


Figure 6 – Phosphatidylcholine structure.

Phospholipids might be natural or synthec. Phosphatidylcholine from natural sources includes soya phosphatidylcholine (SPC) and egg phosphatidylcholine (EPC). Dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) are examples of synthetic phosphatidylcholine (**Figure 7**) [10].

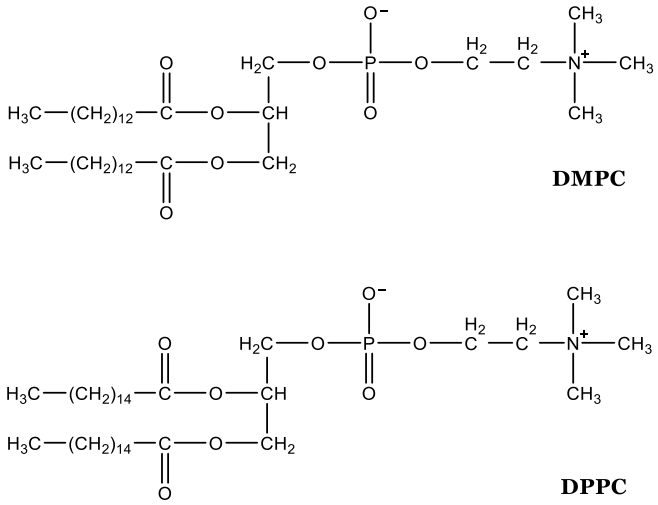


Figure 7 - Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine structures.

The structure and flexibility of lipid bilayers are influenced by the temperature. Below physiological temperatures, the bilayer lipids are in a semisolid gel phase. In this state, the polar head groups are uniformly displayed at the surface and the acyl chains are packed, and with no motion. Above physiological temperatures, the hydrocarbon chains are in constant motion, producing a fluid state, also known as liquid-disordered state, in which the interior of the bilayer is more fluid than solid. At physiological temperatures, lipids are in the liquid-ordered state, where the acyl chains of lipids suffer less thermal motion, but there is a lateral movement in the bilayer plane. The phase transition temperature (T_m) is the temperature at which occurs the transition between the semisolid gel phase and the liquid-disordered state, where the phospholipid bilayer becomes more leaky and flexible. Each type pf phospholipid has a specific T_m. The hydration of phospholipids above the T_m, allows them to assemble into liposomes [10, 17].

Cholesterol, a steroid molecule (**Figure 8**), might be incorporated in liposome bilayers to modify the membrane fluidity, reduce the permeability of water soluble molecules through the membrane, and improve its stability [10]. It functions as a fluidity buffer. Below T_m,

cholesterol makes the membrane more disordered and permeable, while above T_m, it causes membrane organization, stabilizing it [14]. Since the formation of liposomes is achieved by hydration of phospholipids above their T_m, cholesterol enhances the rigidity of liposomes, possibly by filling the gaps between the phospholipid molecules in the bilayer structures [10, 18]. Besides, the presence of cholesterol in the bilayer membranes improves its stability in the presence of biological fluids, such as blood/plasma. In the absence of cholesterol, liposomes tend to react with blood proteins, being destabilized. Cholesterol appears to reduce this interaction. However, the presence of cholesterol does not entirely prevent the loss of liposomal phospholipids [19].

Cholesterol molecules arrange themselves among the phospholipid molecules with the hydroxy group facing towards the water phase and the tricyclic ring trapped between the first few carbons of the fatty acyl chains, into the hydrocarbon core of the bilayer [19].

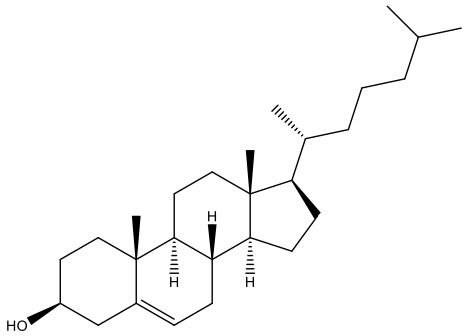
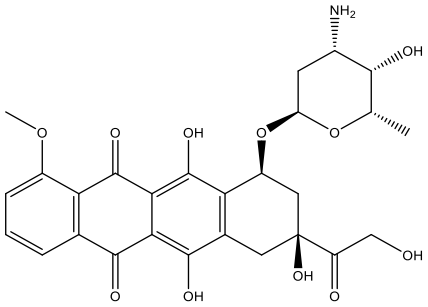
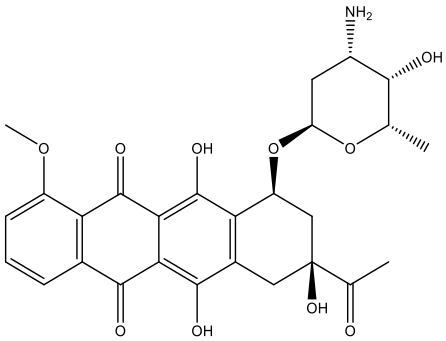
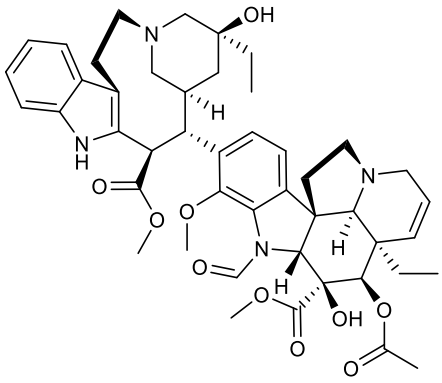


Figure 8 – Chemical structure of the molecule cholesterol.

1.2.2. Liposomes in cancer nanotechnology

In clinical applications, liposomes have proven to take advantage of the EPR effect to passively accumulate in regions of enhanced vasculature permeability, when their average diameter is <200 nm. Thus, the drug-mediated delivery by liposomes explores the overexpression of fenestrations in the cancer vasculature to increase drug concentration in tumor sites. This results in reduced side effects and toxicity of the encapsulated drugs as opposed to free drugs, as well as an increased therapeutic index [4, 11].

Table I- Marketed liposomal products for cancer treatment.

Product	Drug	Indications	Reference(s)
Doxil/Caelyx (Johnson & Johnson) Myocet (Cephalon)	 Doxorubicin	Kaposi's sarcoma; ovarian cancer; breast cancer; multiple myeloma	20-23
DaunoXome (Galen)	 Daunorubicin	Kaposi's sarcoma	24
Marqibo (Talon)	 Vincristine	Acute lymphoblastic leukemia	25-26

Liposomes might also prolong the residence time of drugs in circulation for an effective drug delivery. Cells of the reticuloendothelial system (RES) act as immunological barriers to effective targeting of nanoparticles. The surface of liposomes might be modified with the polymer polyetlylene glycol to avoid the uptake by the RES and increase the circulatory half-time. Therefore, the targeting of liposomes within the tumor is enhanced [4, 9]. Several liposomal products encapsulating drugs have been introduced in the market and many other drugs are in clinical trials. **Table I** shows the approved liposomal products for cancer treatment in the market**.** The parenteral route of administration is predominant for the clinically approved products, in particular intravenous administration [11].

1.2.3. Advantages and drawbacks of liposomes

Liposomes have attracted considerable interest as drug delivery systems since they are versatile drug carriers, suitable for the encapsulation of both hydrophilic and hydrophobic substances. Also, these vesicular structures allow the encapsulation of both small molecules, with the size of an ion, and large molecules of several hundred thousand Daltons [9, 27]. Liposomes present a number of advantages over conventional dosage forms (**Table II**). They have shown to be relatively non-toxic, biodegradable, biocompatible, weakly immunogenic (produce no antigenic or pyrogenic reactions). These properties, like size, charge and surface can be readily handled by the addition of new ingredients to the lipid mixture before the preparation of liposomes or by using different preparation methods. Liposome vesicles act as drug reservoirs, thus protecting drugs from the external environment, such as enzymes and inhibitors. These agents could lead to its inactivation of drugs encapsulated in liposomes. Encapsulation of drugs can increase their stability, avoiding rapid degradation [8-9]. Also, liposome formulations have the ability to control the drug release rate in the presence of biological fluids, retaining a relatively constant and, effective drug concentration in the circulation. Therefore, they can prevent undesirable side effects and reduce drug toxicity [9, 28].

The possibility of targeting liposomes to a particular type of cell or organ leads to the increase of its efficacy and therapeutic index, mainly due to the alteration of biodistribution. Manipulating liposomes for a selective uptake is another way of reducing drug toxicity and injurious side effects because of the minimized drug distribution of the drug. Besides, liposomal drug delivery systems enable the delivery of higher drug concentrations to the

desired target. Moreover, drug encapsulation results in enhanced pharmacokinetic properties, such as reduced elimination or prolonged residence time of the drug in systemic circulation [8-9].

Despite all the advantages of using liposomes as drug delivery systems, this strategy presents a few drawbacks mainly related to their large scale manufacture (**Table II**). There is a necessity to obtain large quantities of the product with reproducible properties and to demonstrate suitable stability during storage and before administration. However, liposomes exhibit poor chemical and physical stability, which restricts its storage for a long period [10, 27, 29-30].

Table II - Advantages and drawbacks of liposomes as drug delivery systems.

Advantages	Drawbacks
- Allows the encapsulation of hydrophilic and hydrophobic drugs, with a wide range of sizes; - Non-toxic, biodegradable, biocompatible, weakly immunogenic; - Easy handle of their properties; - Increased drug stability; - Control of the drug's release rate; - Possibility of directing to a specific target; - Reduced drug toxicity and adverse side effects; - Delivery of superior drug concentrations to the desired target.	- Poor chemical stability; - Poor physical stability; - Lipid exchange between liposomes and HDLs in plasma; - Reduced shelf-life.

Physical instability of aqueous dispersion of liposomes occurs due to vesicle aggregation and fusion, which leads to an alteration in vesicle size and loss of retained material. Problems associated with physical stability of liposomes are important to consider when analyzing the appearance, size and size distribution of liposomes [10, 31].

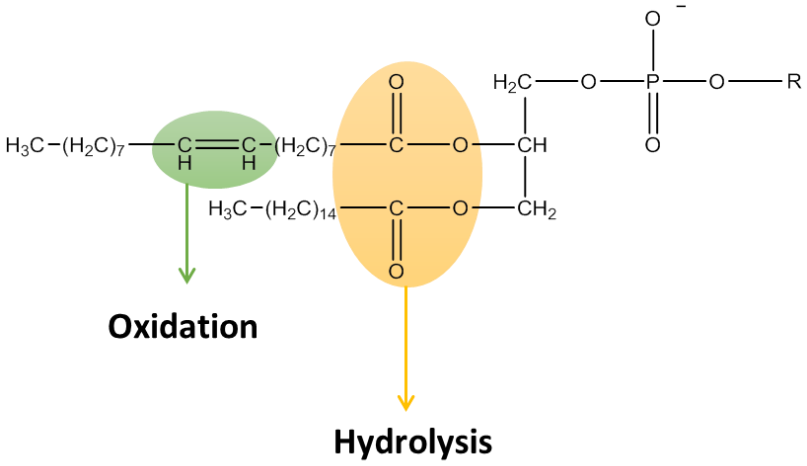


Figure 9 - Representation of molecular moietis of phospholipids associated with the chemical instability of liposomes.

The chemical instability of these systems is caused by the formation of ice crystals in the liposome, which conduces to the destabilization of bilayers resulting in drug leakage. Chemical instability is associated with tendency of phospholipids in liposomal formulations to suffer hydrolysis and oxidation (**Figure 9**). Hydrolysis may occur in ester bonds linking the glycerol backbone to the fatty acids, leading to the disconnection of the hydrophobic chains. In the case of phosphatidylcholine, the hydrolysis might cause the formation of lysophosphatidylcholine (**Figure 10**), increasing the permeability of liposomes.

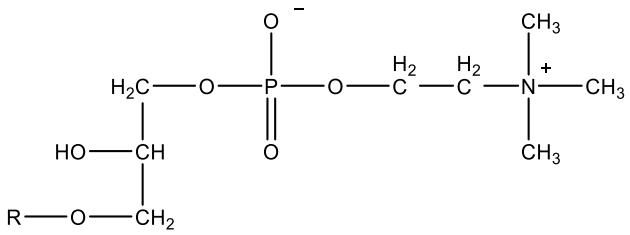


Figure 10 - Structure of lysophosphatidylcholine; **R** = Fatty acid acyl chain.

Therefore, it is important to keep the levels of lyso-phospholipids to a minimum during preparation and storage of liposomes. Oxidation might occur in the presence of unsaturated acyl chains, which could change the permeability of liposomes and their shelf life.

Oxidation of phospholipids might be minimized by protecting them from light or by the addition of antioxidants to the liposomes [10, 19, 31]. Oxidation and hydrolysis of lipids may lead to the formation of short-chain lipids and then less hydrophobic derivatives appear in the bilayers, resulting in compromised quality of liposomes. Besides, the described stability problems cause quicker liposome breakdown and altered drug release profile [32].

In addition, in plasma, liposomes are destabilized due to the lipid exchange between liposome and HDLs, leading to aggregation and leakage of the entrapped material [31].

The refered drawbacks of liposomal formulations (**Figure 11**) limit their clinical application. Indeed, there is a necessity of developing strategies to improve the characteristics of liposomes and, consequently, expanding their applications.

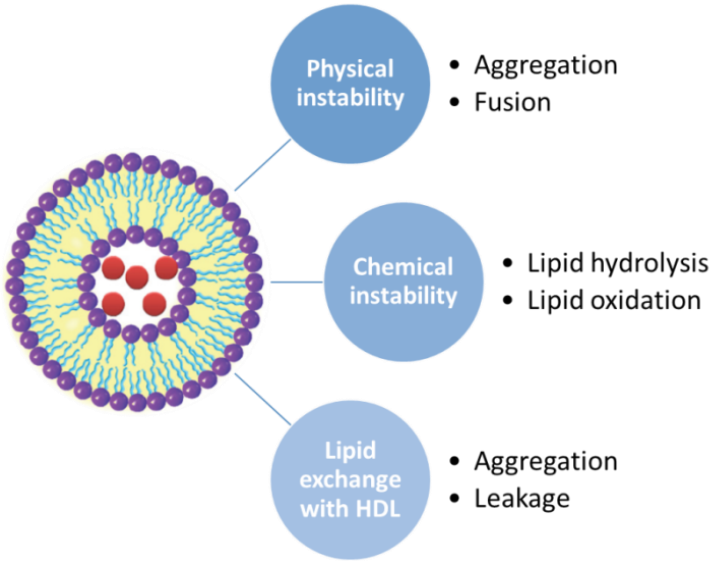


Figure 11 - Possible causes of instability of liposomes.

1.2. Proliposomes

Although liposomes have been widely used for drug delivery, the drawbacks cited above limit their application for medicinal purposes. To be commercialized, these systems need to be stable in the storage time and conditions, and persist intact and active until they reach the biological target. The factors affecting the stability of liposomes will affect their shelf life and their performance *in vivo* [27].

In an attempt to overcome the instability inherent to liposomes, Payne *et al.* introduced, in 1986, a dry phospholipid formulation as an alternative to conventional aqueous liposomes, named proliposome [29-30]. This approach is based on the ability of membrane lipids to form vesicles when they contact with water, enabling the conversion of the proliposome preparation into a liposomal dispersion by addition of an aqueous phase [27, 33].

Originally, proliposomes were defined by Payne *et al.* as dry, free-flowing granular products composed of drug and lipids which form an isotonic multilamellar liposomal suspension, when dispersed in water [30]. However, the concept of proliposomes was expanded, later in 1991, to include liquid phospholipid formulations that can generate liposomes upon addition of aqueous phase [34]. These liquid formulations are concentrated ethanolic solutions of phospholipids. Thus, proliposomes can be generally defined as powdered or liquid lipid formulations that can form liposomes upon addition of aqueous phase and shaking. Usually, powdered formulations are better suited to entrap lypophilic drugs because the greater part of drug locates into the liposomal lipid phase. Liquid lipid formulations are suitable for the entrapment both hydrophobic and hydrophilic drugs [10]. The application of proliposomes is extended to several administration routes [35-39].

In this work, attention will be given only to powdered lipid proliposomal formulations. Powdered proliposomes are composed of a carrier which is a water soluble porous powder, usually the polyol sugars mannitol or sorbitol (**Figure 12**), where phospholipids and the drug dissolved in organic solvent might be loaded [28].

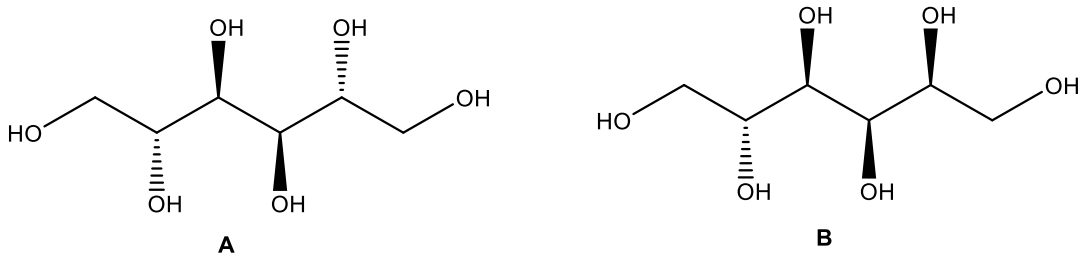


Figure 12 - Mannitol (**A**) and sorbitol (**B**) structures.

Sterilized proliposomes can be stored in a dry state and then dissolved in aqueous solution to form a liposomal suspension when necessary [28]. The conversion of proliposomes into liposomes may take place *in vivo* by the effect of physiological fluids, or *in vitro* before the administration, by addition of a convenient hydrating fluid above the T_m of the lipid, followed by shaking [10, 27].

Drying an organic solution of phospholipids and a carrier/drug results in the formation of particles involving a crystalline carrier/drug at the core encapsulated by a phospholipid shell [40]. When an aqueous phase is added to proliposomes, the process of dissolution/disintegration might occur by a progressive hydration of the lipid surface of proliposome. Then, liposomes "bud off" from the central core of the proliposome until hydration of the lipid and carrier dissolution is complete [29].

The fact that proliposomes are accessible in a dry powder form makes them easy to distribute, transfer, measure and store and consequently, a useful and economic delivery system [10, 27]. Besides, proliposomes demonstrated controlled drug release, improved stability and increased solubility relatively to conventional liposomes [31]. Preparing liposomes by conversion from proliposomes allows the encapsulation of a wide variety of drugs with different solubility in water and organic solvents and presents high encapsulation efficiencies when compared to other methods based on passive entrapment [33].

Several studies of drug incorporation in proliposomes revealed that these systems have the ability to convert a drug from its crystalline form to its amorphous state [35, 41-42]. It is established that drugs in amorphous state have higher solubility. In this state, thermodynamically unstable, since no energy is required to break up the crystal lattice while the dissolution process occurs, the drug release is enhanced when in that physical state [43-44].

1.3.1. Manufacturing processes

From the variety of manufacturing processes of powdered proliposomes, attention will be given to film deposition on carrier, freeze drying and spray drying.

1.3.1.1. Film deposition on carrier

This is the original method used by Payne *et.al* to produce proliposomes, which requires a modified rotary evaporator (**Figure 13**) [30]. It involves a film of drugs and phospholipids, which is deposited onto a porous, water soluble carrier material [10].

The selection of the carrier material is of great importance for formulation of powdered proliposomes by film deposition on carrier. It should be selected based on its solubility, porosity and ability to accommodate phospholipids on its surface. The particle size of the carrier influences the size and polydispersity of the generated liposomes [18].

The process involves two main steps. First, the selected carrier is placed in a round-bottom flask, attached to a rotary evaporator and dried under reduced pressure [45-46]. In the second step, a solution of drug and phospholipids in a volatile organic solvent is sprayed dropwise, from a separating funnel to a bed of carrier material, via feed tube (**Figure 13**) [47-48]. Thus, a thin film of phospholipid is coated onto the carrier surface and the organic solvent is evaporated to obtain a dry granular material [10, 27]. The goal of modifying the rotary evaporation unit is to ensure the efficient mixing of the formulation components and to monitor the temperature of the powder bed [30].

Preparation of proliposomes using film deposition on carrier method is one of the most cost-effective strategies to produce liposomes in large scale. Liposomes formed by this method have shown to be similar to conventional liposomes [31]. However, the step of the addition of the organic solution to the rotary evaporator and its evaporation makes this method slow and difficult to control [27].

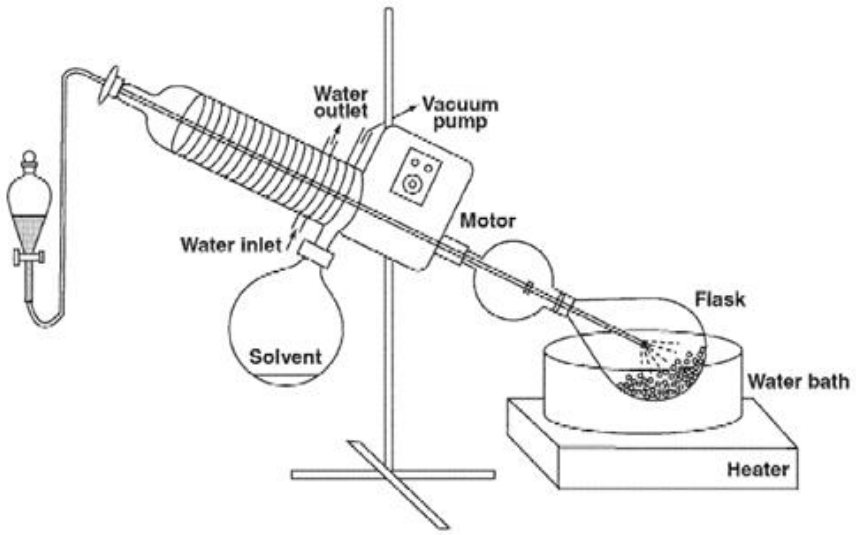


Figure 13 - Apparatus used to prepare proliposomes by film deposition on carrier (from [49]). The reproduction of this figure was authorized.

1.3.1.2. Freeze drying

Freeze drying, also called lyophilization, implicates heat and mass transfer to and from the product that is being prepared. To use this technique, a solution of drug, lipids and a carrier material is placed in a freeze dryer, where the procedure occurs. This comprises three stages: the freezing stage, consisting in cooling the solution until it is frozen; a primary drying stage, in which the frozen solvent is removed by sublimation by action of the vacuum; and a second drying stage to remove the solvent that did not freeze [44, 50-51].

For the success of sublimation step, it is fundamental the avoidance of ice fusion. This may be achieved as long as one operates below the triple point of the pressure-temperature equilibrium of water, which occurs near the temperature 0 ^{o}C and at the absolute pressure of 4,59 mm Hg (**Figure 14**). At the triple point, water coexists simultaneously in solid, liquid and gaseous states. Below the triple point, it only coexists in the solid and the gaseous states (**Figure 14**), allowing the direct evaporation of the frozen particles, bypassing the liquid state of the water [44, 50-51].

Freeze drying allows the preservation of chemical properties of substances that might be affected by heat for a long period, since the procedure occurs at low temperatures. Also, using freeze drying, the drug is subjected to a minimal thermal stress during the solid particles formation, minimizing the risk of phase separation as soon as the solution is vitrified. Besides, lyophilized products have a sponge structure, allowing their rapid dissolution [44, 50]. However, freeze drying is time-consuming and expensive [10].

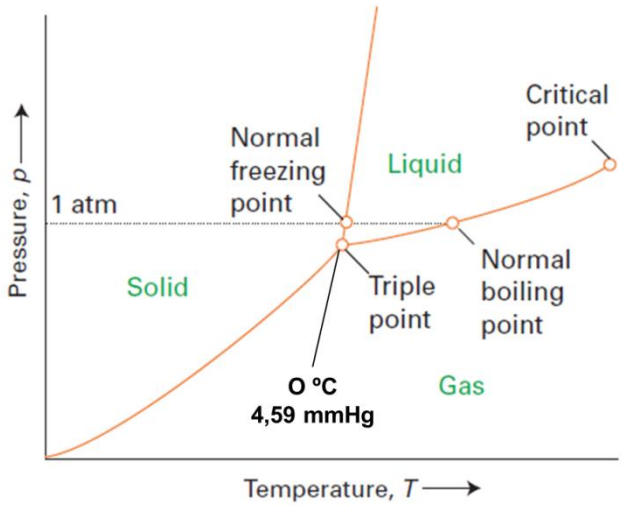


Figure 14 - Pressure-temperature equilibrium diagram. At the triple point, solid, liquid and vapor are in dynamic equilibrium. Liquid-vapor phase limit ends at the critical point. The normal freeze point is the temperature at which the liquid freezes at a pressure of 1 atm and the normal boiling point represents the temperature at which the liquid vapor pressure is 1 atm. Adapted from [52].

Freeze drying has already been employed for the drying of liquid liposomal formulations with a cryoprotectant, usually a carbohydrate molecule, in order to increase their stability. Minimizing the levels of residual water can improve the shelf-life of lyophilized liposomes and prevent the increase of vesicle size upon rehydration [53-54]. Besides, when the method of the film deposition on carrier is employed to produce proliposomal powders, some residual organic solvents may still remain in the formulation and freeze drying might be used to complete the drying of the powder product [47, 55-56]. Also, Fei *et al.,* reported the use of freeze drying to directly produce proliposomes [57].

1.3.1.3. Spray Drying

Spray drying has a great utility in pharmaceutical industry, mainly in the production of solid dispersions [43, 58], and recently, has also been explored to produce proliposomes for the pulmonary delivery of drugs [38, 59-61]. Spray drying is characterized by its ability to comprise in one step the formation of particle and its drying, which allows a better control of the particle formation [18].

Spray drying converts liquid feed of the drug, which can be a solution, emulsion or suspension, into a dry powder [18]. Spray drying involves the addition of the carrier material, commonly a sugar, to an organic solution comprising the drug and lipids, and its subsequent spraying into a stream of heated air flow to remove the solvent [10, 58-59, 62]. This process involves four stages (**Figure 15**):

1) Atomization of the product into a spray nozzle;
2) Spray-air contact;
3) Drying of the spray droplets;
4) Collection of the solid product.

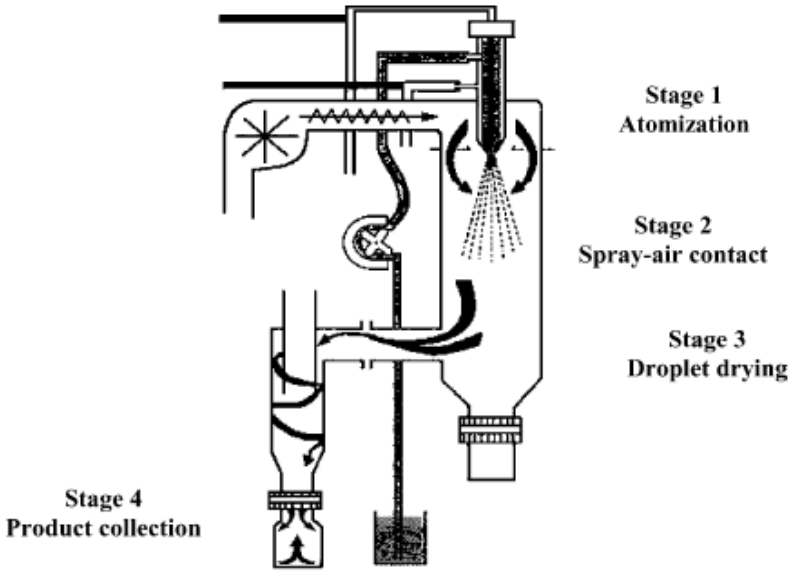


Figure 15 – Schematic representation of the stages in the spray drying technique (from [63]). The reproduction of this figure was authorized.

The solutions subjected to spray drying are pumped and atomized into the drying chamber with a spray nozzle, and dried in a concurrent heated air flow that is later collected in a reservoir [43, 63]. The large surface area of the droplets and the high temperature of the drying air leads to the rapid solvent evaporation and formation of the solid dispersion in seconds, which might be fast enough to prevent phase separation and to allow thermolabile molecules to be converted into fine powder [10, 58].

Spray drying parameters exert a great influence in the powder characteristics, such as particle size, size distribution, shape, morphology and density, which might be optimized since spray drying has the ability to manipulate and control a variety of parameters, including the solvent composition, solute concentration, feed rates of solution and gas, temperature and droplet size [10, 18].

Spray drying usually produces drugs in the amorphous state, but occasionally the drug may be partially crystallized during processing [62].

Spray drying allows the production of a fine, dust free powder as well as agglomerated to particular specifications, within a narrow range of particles sizes, which makes it especially advantageous when it is necessary to prepare particles of uniform size and shape. Also it is cost effective, which makes it easy to scale up and suitable for both laboratory and industrial scale production of proliposomes [27, 43, 64].

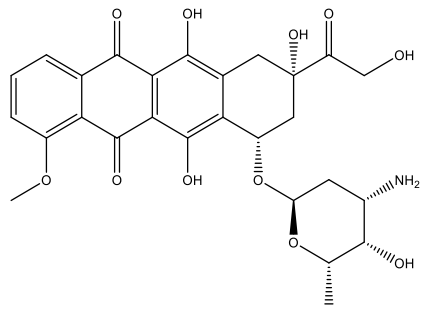
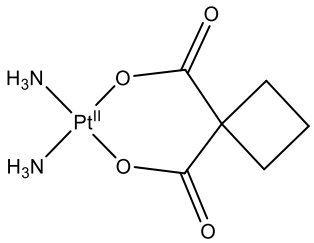
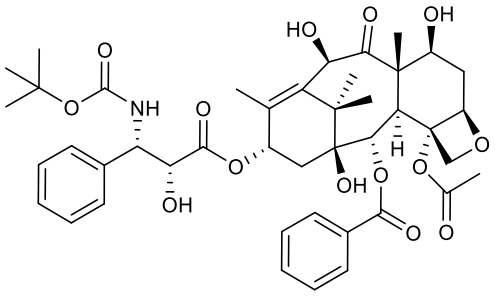
Spray drying has also been used for the drying of liposomal formulations. Spray-dried liposomes of the drugs verapamil and metronidazole with mannitol as the carrier preserved the size distribution of liposomes and entrapment efficiency of the drug after a year of storage [65].

1.3.2. Proliposomes and anticancer drugs

It is known that the intravenous route is the preferred route for the administration of liposomes as drug delivery systems. The studies of proliposomal formulation to encapsulate anticancer drugs have also been applied to this route of administration. The anticancer drugs studied for encapsulation in proliposomal formulations are presented in **Table III**. The studied proliposomes exhibited good stability for at least 12 months [66]. The encapsulation of anticancer drugs in liposomes after hydration of proliposomes seems to alter the

pharmacokinetic and tissue distribution profiles and increase the therapeutic efficiency when compared to an intravenous administration of the free drug [37, 67].

Table III - Anticancer drugs encapsulated in proliposomes.

Drug	Indications	Reference(s)
 Adriamycin	Solid tumors; malignant lymphomas; acute leukemia	68-69
 Carboplatin	Ovarian cancer; small or non-small cell lung cancer; head and neck cancer; lung cancer	67
 Docetaxel	Non-small cell lung cancer	37, 66

1.3. Xanthones

Chemically, xanthones are a class of oxygenated heterocyclic compounds with a dibenzo-γ-pyrone scaffold (**Figure 16**) [70-72].

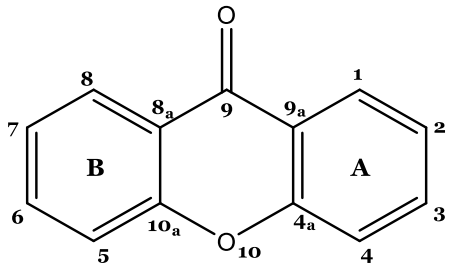


Figure 16 - Xanthone scaffold (numbered according IUPAC).

Although xanthone (9*H*-xanthen-9-one) does not occur in nature, xanthone derivatives are frequently isolated as secondary metabolites from plants and microorganisms [71, 73]. These oxygenated heterocycles are structurally related to other natural compounds with the γ-pyrone scaffold: flavonoids (**Figure 17A**) and chromones (**Figure 17B**) [71].

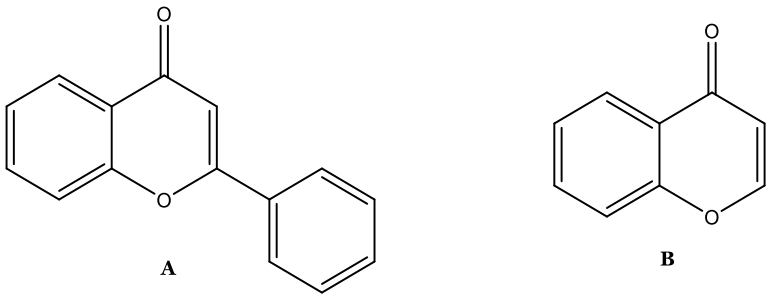


Figure 17 - Scaffolds containing a γ-pyrone moiety: flavonoids (**A**) and chromones (**B**).

Xanthones derivatives from natural sources have attracted great interest due to associated large variety of pharmacological activities [71]. However, naturally-occurring xanthones present a relatively restricted structural diversity since the biosynthetic pathways limit the type and position of the substituents in the xanthone scaffold. Naturally occurring

xanthone derivatives are subdivided into six major groups: simple oxygenated xanthones, glycosylated xanthones, prenylated xanthones and their derivatives, xanthone dimers, xanthonolignoids and miscellaneous, depending on the nature of the substituents in the dibenzo-γ-pyrone scaffold [74]. The use of chemical synthesis expanded the possibilities of having substituents from different nature and in different positions on the xanthone scaffold. By chemical synthesis, simple functional groups such as hydroxyl, methoxyl, methyl and carboxyl have been introduced in the xanthone nucleus, as well as more complex substituents such as epoxide, azole, aminoalcohol, sulfamoyl and dihydropyridine, and others [74]. Xanthones can be considered as "privileged structures", considering that their scaffold contains a rigid ring system allowing the insertion of a variety of substituents capable of interacting with several biological targets [75].

Among the diversity of biological activities described for natural and synthetic xanthones, the *in vitro* growth inhibitory activity on tumor cell lines seems to be quite important, since they exert their effect on a wide range of different tumor cell lines [74, 76-77].

Although xanthone derivatives present a broad spectrum of biological activities, most of the substituents introduced (by nature or by synthesis) confer poor aqueous solubility, which is often linked with poor bioavailability [78]. Considering this, micro and nanoparticulate systems present an interesting strategy to overcome this solubility problem. In CEQUIMED-UP, both natural and synthetic xanthone derivatives have been encapsulated in micro and nanoparticles containing biocompatible and biodegradable polymers [79-86]. Xanthone derivatives incorporated in polymeric microparticles and nanoparticles, and their respective biological activities are presented (**Table IV**).

Table IV - Xanthone derivatives encapsulated in micro and nanoparticles.

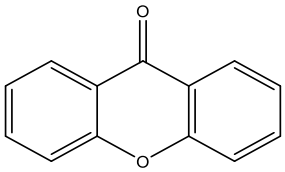
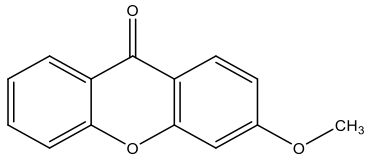
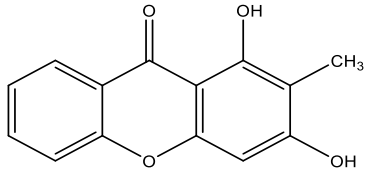
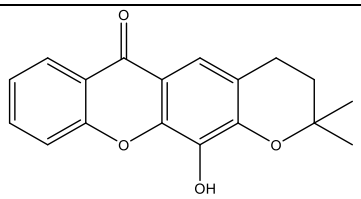
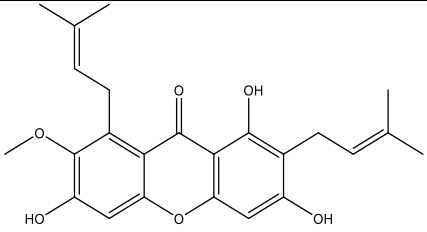
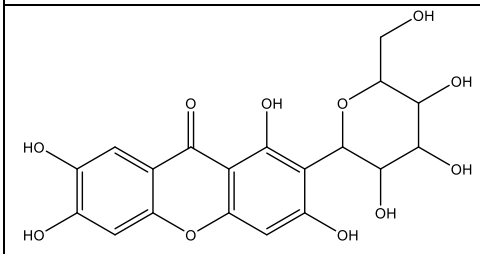
Xanthone derivative	**Biological activity**	**Type of particles**	**Reference(s)**
 **Xanthone** (synthetic)	Monoamine oxidase inhibitor; protein kinase C activator; inhibitory effect on NO production [83-84, 87]	Polymeric nanoparticles	[81, 83-84]

Table IV (cont.)

 **3-Methoxyxanthone (**synthetic)	Monoamine oxidase inhibitor; protein kinase C activator; inhibitory effect on NO production [83-84, 87]	Polymeric nanoparticles	[81, 83-84]
 **1,3-Dihydroxy-2-methylxanthone (**synthesic)	Inhibitory effect on the growth of a human breast cancer cell line [79]	Polymeric nanoparticles	[79]
 **3,4-Dihydro-12-hydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-b]xanthen-6-one** (synthesis)	Antiproliferative effect on a human breast cancer and leukemia cell lines; apoptotic effect in leukemia cell lines [88-89]	Polymeric nanoparticles	[80]
 **α-Mangostin** (natural)	Antioxidant; neuroprotective; antimicrobial; antiprotozoal; vasorelaxant [90]	Polymeric microspheres	[91]
 **Mangiferin** (natural)	Antioxidant; anti-allergic; anthelminthic; gastro-protective; antitumor; antiviral [82]	Polymeric microparticles	[82]

In the present work, a carbaldehyde xanthone derivative, **LEM2** (**Figure 18**), which was tested for antitumor effect and displayed activity in breast cancer (MCF-7), melanoma (UACC-62) and glioma (SF268) cell lines (unpublished results), was synthesized *in house* in suitable amount to be incorporated into proliposomal formulations.

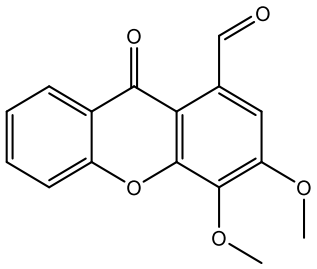


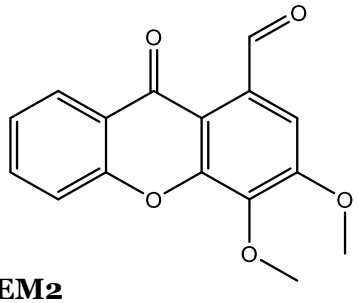
Figure 18 - LEM2 structure.

CHAPTER 2

AIMS

CHAPTER 2 – AIMS

LEM2 is a synthetic xanthone with a very promising inhibitory effect on the growth of breast cancer, melanoma and glioma cell lines.



LEM2

Cancer is among one of the leading causes of death worldwide and the treatment present a number of associated problems, that nanotechnology has been recently trying to overcome. Cancer nanotechnology has emerged as a promising strategy for the therapy of cancer diseases. Nanosystems might be rational designed to achieve the desired concentration of the drug in tumor sites and minimize the distribution to the normal cells.

Among the nanosystems, liposomes have adequate properties for drug delivery in cancer treatment and several liposomal products with anticancer drugs have been approved and are already in the market.

Although liposomes present good properties as drug delivery systems, they have poor chemical and physical stabilities, which could limit their shelf life and performance *in vivo*. The proliposome strategy arises as a mean to overcome the instability inherent to liposomes.

Based on these considerations, the main objectives of the present dissertation are:

- Synthesize the carbaldehyde xanthone derivative **LEM2** with a very promising inhibitory effect on the growth of breast cancer, melanoma and glioma cell lines in adequate amount to incorporate into proliposomal formulations;
- Develop an HPLC method for the quantification of **LEM2** in proliposomal formulations;

- Develop and optimize proliposomal formulations by different methodologies, using:
    - Egg phosphatidylcholine
    - Cholesterol
    - Mannitol
    - LEM2
- Evaluate size, morphology and physical state of proliposomal powders obtained from the different methodologies;
- Evaluate size, zeta potential, entrapment efficiency and morphology of liposomes obtained by hydration of solid proliposomes;
- Evaluate the stability of proliposomes.

CHAPTER 3

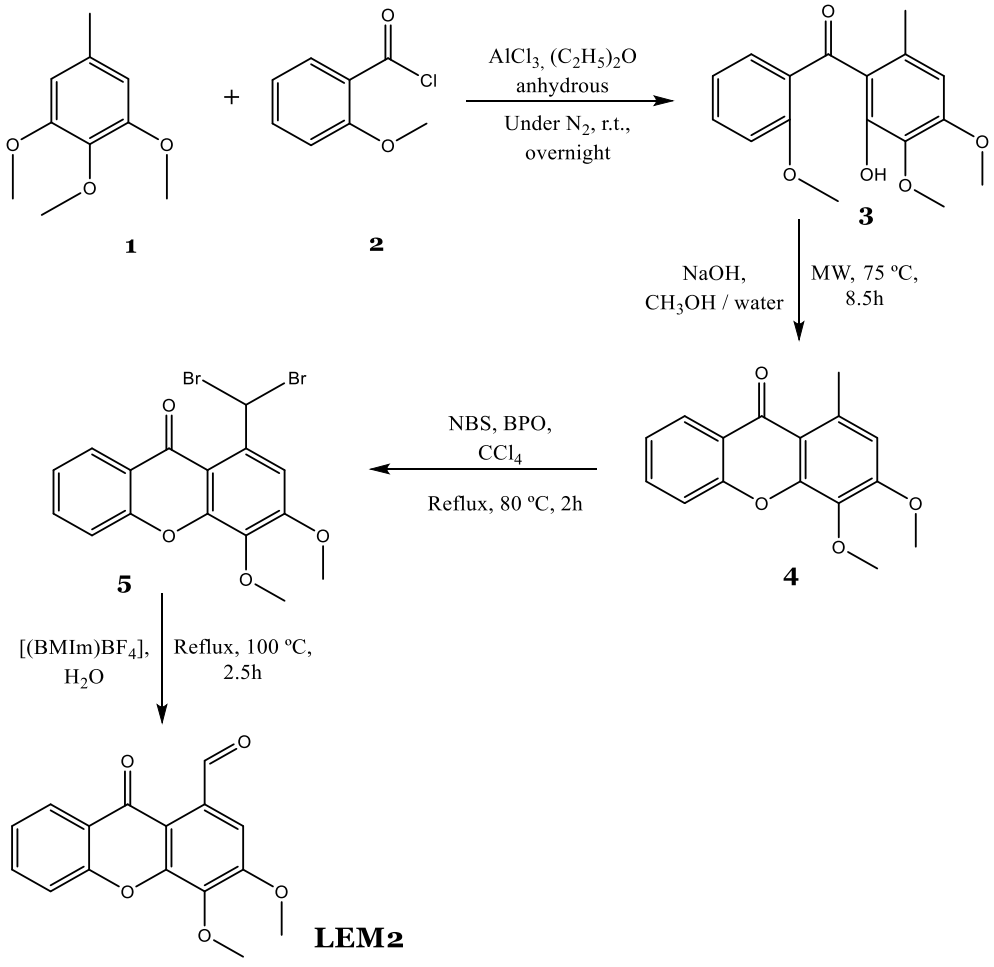
RESULTS AND DISCUSSION

CHAPTER 3 – RESULTS AND DISCUSSION

3.1. Synthesis of the carbaldehydic xanthone derivative LEM2

LEM2 is a synthetic carbaldehydic xanthone derivative, with poor solubility in water, which was tested for its antitumor effect and displayed activity in breast cancer (MCF-7), melanoma (UACC-62) and glioma (SF268) cell lines (unpublished results).

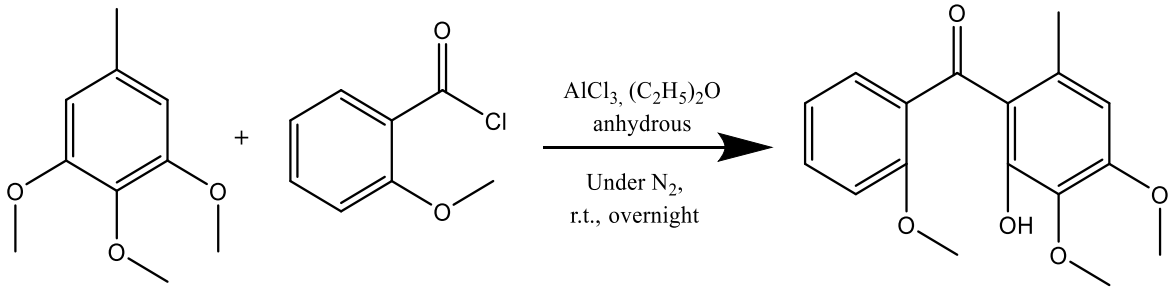
The synthesis of **LEM2** was performed according to previous studies and is depicted in **Scheme 1** [92].



Scheme 1 - General synthesis of **LEM2**. r.t. = room temperature; MW = microwave; NBS = N-bromosuccinimide; BPO = benzoyl peroxide; [(BMIm)BF_4] = 1-butyl-3-methylimidazolium tetrafluoroborate.

3.1.1. Synthesis of benzophenone intermediate 3, (2-hydroxy-3,4-dimethoxy-6-methylphenyl) (methoxyphenyl) methanone

The synthesis of some kind of xanthones can be achieved using benzophenone intermediates as precursors. Benzophenone intermediates suitable for cyclisation to xanthones may be synthesized by Friedel-Crafts acylation of methoxybenzene derivatives with an appropriate substituted benzoyl chloride in the presence of a Lewis acid, as aluminium chloride, in ether, at room temperature (**Scheme 2**) [93].



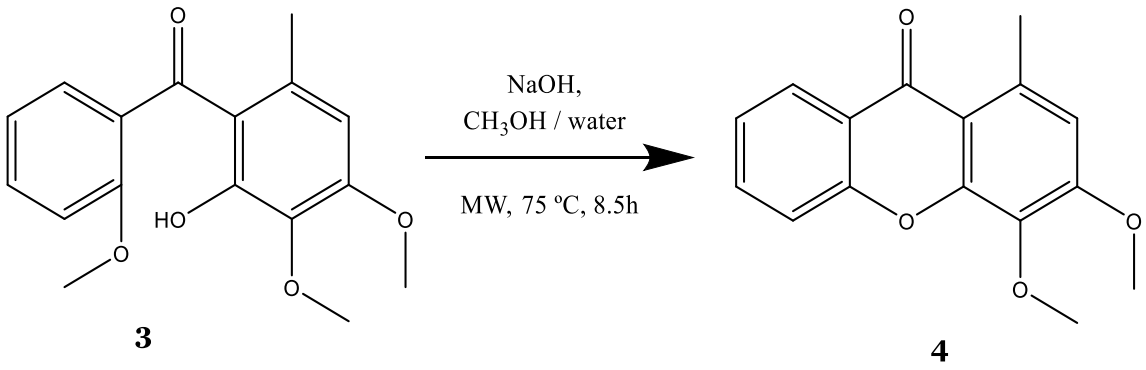
1 **2** **3**

Scheme 2 - Friedel-Crafts acylation of 3,4,5-trimethoxytoluene (**1**) with 2-methoxybenzoyl chloride (**2**) to obtain the benzophenone intermediate 3. r.t. = room temperature.

To obtain benzophenone intermediate **3**, aluminium chloride was added to a dry diethyl ether solution of 2-methoxybenzoyl chloride (**2**) and 3,4,5-trimethoxytoluene (**1**). The reaction occurred under N_2, resulting in a deep red mixture. After completing the reaction, the suspension was acidified with HCl (5M) to convert the phenolate group of the benzophenone derivative (**3**) to a non-ionized form. Then, the non-ionized form of the benzophenone derivative was extracted with chloroform. After drying with anhydrous sodium sulphate and concentrate of the organic layer, brown oil was obtained which contains the final product. The identity of intermediate **3** was confirmed by TLC comparing with standard compound previously obtained [92]. The obtained oil (327,8 mg) was used without further purification processes for the next reaction step.

3.1.2. Synthesis of 3,4-diethyl-1-methyl-9*H*-xanthen-9-one (4): cyclization of benzophenone intermediate 3

The benzophenone intermediate **3** in the crude product from the first reaction was the precursor for cyclisation to the xanthone intermediate (**Scheme 3**).



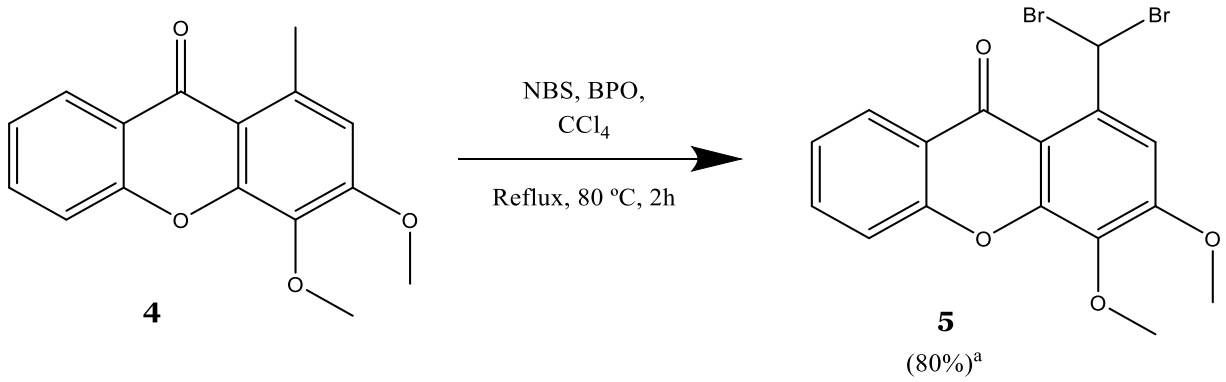
3 **4**

Scheme 3 - Cyclization of the benzophenone intermediate **3** to xanthone intermediate **4**. MW = microwave.

The cyclization of benzophenone intermediates under conventional heating is time-consuming. Hence, it was preferred the use of microwave heating instead of conventional heating to obtain the xanthone intermediate **4**, in order to reduce the reaction time [94]. The reaction of cyclization was undergone in basic medium, which contributes to the increase of the acidity of the phenolic hydroxyl group, leading to a nucleophilic attack to the carbon directly bounded to OCH_3 and subsequent elimination of methanol. The reaction product contained a white solid, insoluble in basic solution, containing the xanthone intermediate **4**. After completing the reaction, the solid was trapped by a vacuum filtration and after that extracted with dichloromethane. The intermediate **4** was present in the organic layer from the extraction. The solutions containing the xanthone intermediate **4** were dried, and 3,093 g were obtained. The identity of intermediate **4** was confirmed by TLC comparing with the standard compound previously obtained [92].

3.1.3. Synthesis of 1-(dibromomethyl)-3,4-dimethoxy-9*H*-xanthen-9-one (5)

The dibromated xanthone intermediate **5** can be synthetized via Wohl-Ziegler reaction, by a benzylic bromination of xanthone intermediate **4** (Scheme **4**). Benzoyl peroxide (BPO) acts as a catalyst since its oxygen-oxygen bond is weak, allowing BPO to easily undergo homolytic cleavage, providing reactive free radicals to initiate the reaction of benzylic bromination. *N*-bromosuccinimide (NBS) is a source of bromine radicals, largely used for the Wohl-Ziegler reaction [95].

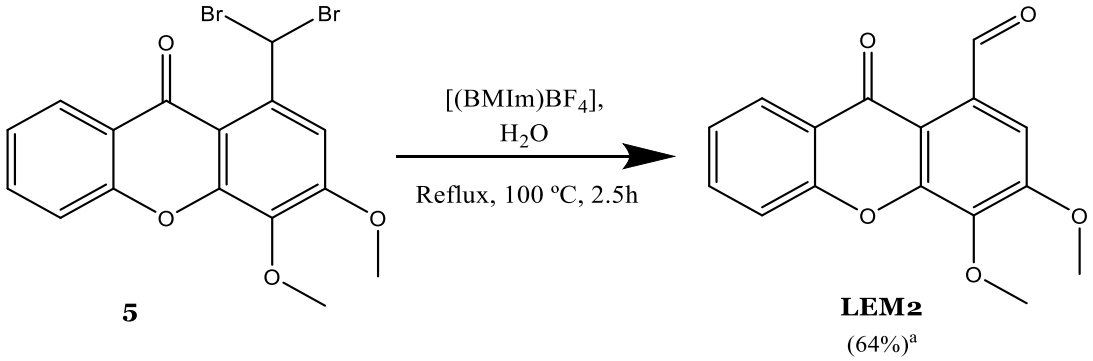


Scheme 4 - Wohl-Ziegler reaction of dibrominated xanthone intermediate 5 from xanthone intermediate **4**. NBS = N-bromosuccinimide; BPO = benzoyl peroxide. a isolated yield.

The reaction was performed using carbon tetrachloride as solvent. After completing the reaction, some insoluble impurities were trapped by vacuum filtration. The TLC analysis revealed that the dibrominated xanthone intermediate **5** was exclusively present in the mother liquor and that some impurities were still present. Therefore, the mother liquor was submitted to a flash column chromatography and the fractions containing the intermediate **5** where collected. However, those fractions were found to contain some impurities. Consequently, a chromatography flash cartridge was used as an additional purification step. The identity of intermediate **5** was confirmed by TLC comparing with the standard compound previously obtained [92]. The dibrominated xanthone intermediade **5** was obtained with a yield of 80%.

3.1.4. Synthesis of 3,4-dimethoxy-9-oxo-9*H*-xanthene-1-carbaldehyde (LEM2)

After the synthesis of dibrominated xanthone intermediate **5**, it was needed to perform the debromination reaction in order to accomplish the oxidation to the carbaldehydic xanthone **LEM2**. For that, an ionic liquid was used, which is a promising strategy for debromination. The ionic liquids are classified as green solvents and have been widely explored in organic chemistry for the last few years [96]. For α-dibromoketones, the use of the ionic liquid 1-methyl-3-pentylimidazolium tetrafluoroborate [(pmIm)BF_4] under MW irradiation can produce either monobromoketones or doubly debrominated ketones [97]. However, in the present study, the intermediate **5** is a ɣ-dibromoketone and the reaction occurred under conventional heating (100 ºC) using the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate [(BMIm)BF_4] to afford **LEM2** directly by a solvolytic displacement of dibrominated xanthone intermediate **5** using water and ionic liquid as solvents (**Scheme 5**).



Scheme 5 - Synthesis of carbaldehydic xanthone derivative **LEM2** from xanthone intermediate **5**. [(BMIm)BF4] = 1-butyl-3-methylimidazolium tetrafluoroborate. a isolated yield.

In the course of the reaction, an intense spot with a different Rf of the xanthone intermediate **5** was appearing in TLC plates, while the spot corresponding to intermediate **5** progressively disappeared. After finishing the reaction, the crude material was submitted to a liquid-liquid extraction with ethyl acetate in order to remove the ionic liquid and the water used in this reaction. The organic layer was dried and submitted to a chromatography flash

cartridge to remove the impurities contained on the crude material. The resulting pure fractions were collected and concentrated. However, some impure fractions were obtained, which were concentrated and crystalized from ethyl acetate/*n*-hexane. **LEM2** purity and identity was confirmed by TLC based on comparison with the reference sample previously obtained *in house* [92]. The synthesis of xanthone **LEM2** was successfully completed with an isolated yield of 64%.

3.2. Development of an HPLC method for the quantification of LEM2

To quantify **LEM2** in proliposomal formulations, a methodology using High Performance Liquid Chromatography (HPLC) equipped with an UV detector was used. The UV spectrum of **LEM2** was performed, with the wavelength ranging from 200 to 400 nm (**Figure 19).** The maximum absorption of **LEM2** occurs at the wavelength 242 nm, which was the wavelength chosen to detect the compound by the HPLC method.

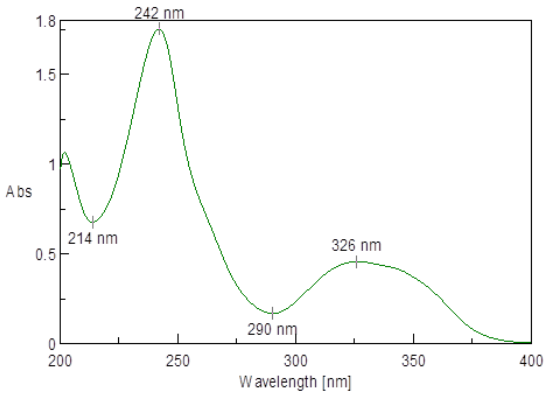


Figure 19 - UV absorption spectrum of LEM2.

Calibration curves were prepared and analyzed in triplicate on three different days in a isocratic elution with the mobile phase water : methanol (25:75) at a flux of 1.0 ml/min. The **Table V** shows the concentration of **LEM2** standard solutions and respective medium peak areas obtained from the chromatogram of **LEM2**. Data were fitted to least squares linear

regression and a calibration curve was obtained (**Figure 20**), with the respective equation of the curve (**Equation 1**) having a correlation coefficient (R) of 0.9994. The essay demonstrated good linearity in the tested range for **LEM2** (R^2 = 0.9989) [98].

$$y = 0.4281x - 0.0327 \qquad \textbf{Equation 1}$$

Table V - Concentration of **LEM2** standard solutions and respective peak areas.

Concentration (µg/ml)	Peak area
0.25	0.0914
0.50	0.211
1.00	0.378
2.00	0.793
3.00	1.223
4.00	1.717
5.00	2.072
6.00	2.566

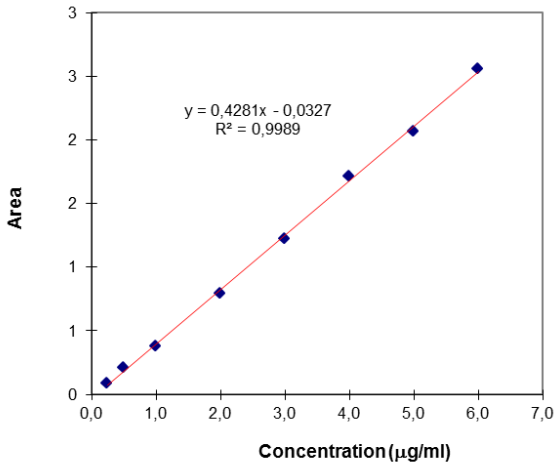


Figure 20 –Calibration curve to extrapolate LEM2 concentration values using HPLC method.

Figure 21 shows that the standard deviation of the concentration values of **LEM2** standard solutions are randomly dispersed, reinforcing the linearity of the method.

Figure 22 shows that for the response / concentration relation, most of the points are within the value of mean ± 5%. Although, two of the points exceed this value, they fit within the ± 10% limit, supporting the linearity of the method.

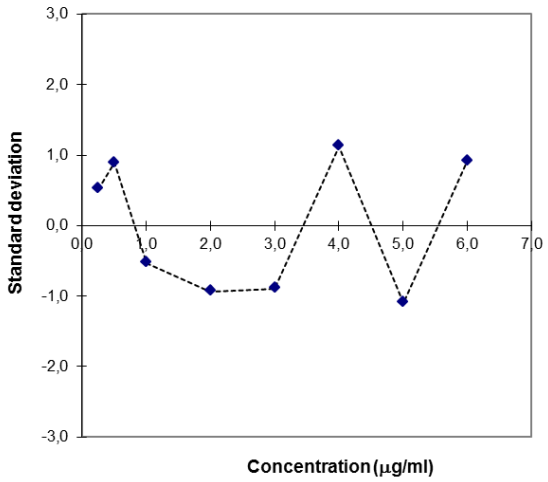


Figure 21 – Graphic of the standard deviation of concentration values of **LEM2** standard solutions.

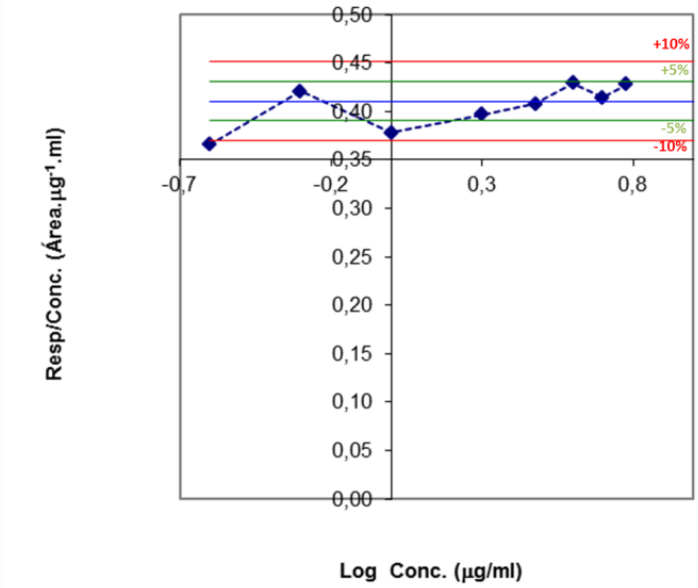


Figure 22 - Graphic of response / concentration vs concentration logarithm.

Figure 23 shows a typical chromatogram of **LEM2** standard solutions with the retention time about 7.3 min for **LEM2**.

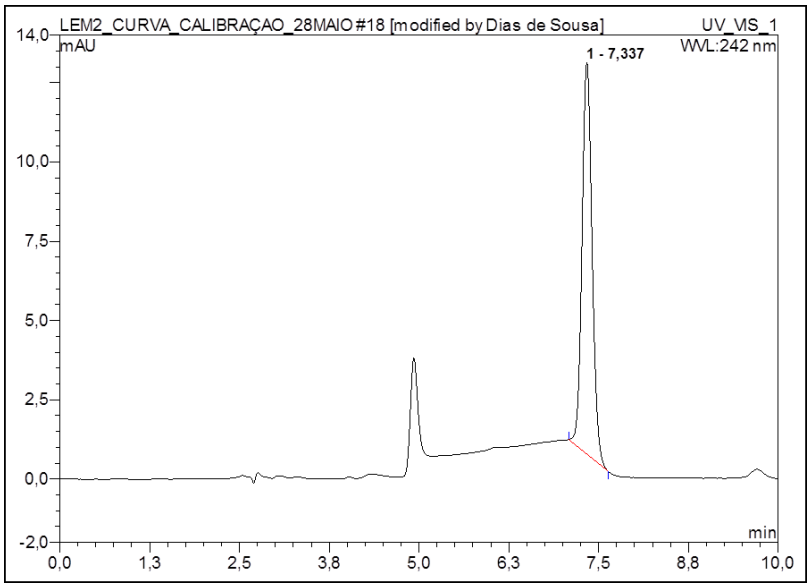


Figure 23 – LEM2 standard solution chromatogram.

Figure 24 proves the specificity of the analytical method for **LEM2**, since the UV spectrum obtained from the chromatogram throughout the presented peak, is superposed to the **LEM2** UV spectrum (**Figure 19**).

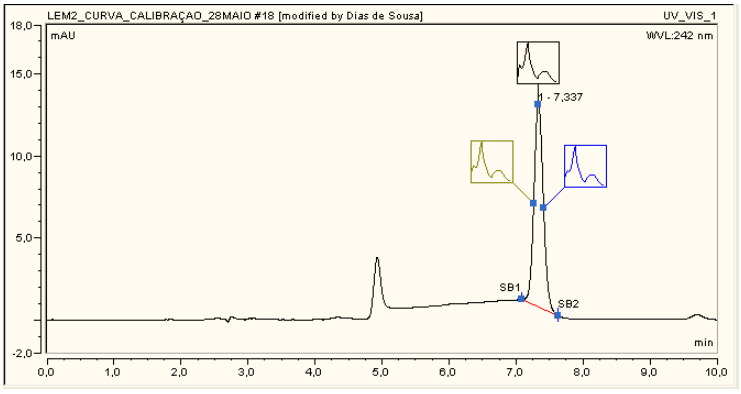


Figure 24 – LEM2 standard solution chromatogram and UV spectrum at 242 nm for the specific detection of the compound.

3.3. Preliminary studies for the development of proliposomal formulations

Proliposome formulations are comprised of a lipid part and a carrier material. For this study, the lipid part was composed by a mixture of egg phospholipids with 80% of phosphatidylcholine (PC) and cholesterol (CH), and the carrier material used was mannitol. Phospholipids from natural sources have lower costs than synthetic phospholipids. Egg and soybean are the principal sources of natural phospholipids for production of liposomes, and their phase transition occurs at negative temperatures. EPC liposomes present lower leakage rates and higher saturation levels than SPC liposomes, which improves their oxidative stability [99]. These factors are important to consider when producing proliposomes, since the main purpose proliposomes is to enhance the stability of liposomes. Cholesterol has the ability to enhance the rigidity of liposomes and improve the stability of lipid bilayers in the presence of biological fluids, as stated above [10, 18-19]. Mannitol is a water soluble sugar, and a frequently used carrier material for proliposomal formulations, allowing the production of dried powders with loose appearance [46].

A preliminary study was performed to choose the molar ratio between egg phosphatidylcholine and cholesterol to continue the proliposomal formulation studies.

Using the method film deposition on carrier, the molar ratio between PC and CH was varied, keeping constant the weight ratio between carrier material (mannitol) and the lipid (5:1). The molar ratio PC : CH (3:1) was chosen, proliposomes were hydrated with water and their characterization is presented in **Table VI**.

Table VI - Characterization of liposomes formed from the proliposomal formulation PC : CH (3:1) produced by film deposition on carrier.

Effective diameter (nm)	Polydispersity	Zeta potential (mV)
621.4 ± 31.0	0.341 ± 0.025	-58.46 ± 7.42

Previous studies stated that the amount of carrier material in proliposomal formulations is a determinant factor on the properties of powder particles [38, 59-60]. Thus, the weight ratio between the carrier material and the lipid part was studied, by freeze drying, keeping the molar ratio PC : CH (3:1). The liposomes reconstituted from hydration of proliposomes are characterized in **Figure 25** and **26** (and **Appendix I**).

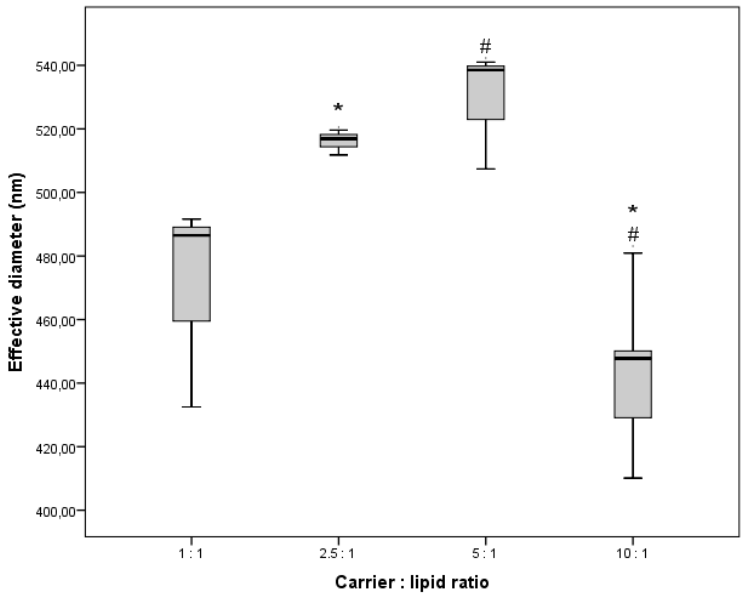


Figure 25 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by freeze drying, with different carrier : lipid weight ratio. Each box represents three individual batches. Statistical significance: * $p < 0.009$, # 0.003.

The effective diameter of liposomes seems to insignificantly increase when the lipid : carrier weight increases from (1 : 1) to (5 : 1) (**Figure 25**). However, for the ratio (10 : 1), the effective diameter significantly decreases comparing to the ratios (2.5 : 1) and (5:1). This could be explained by the fact that the presence of the carrier material prevents in some extent the interaction between the lipids. So, increasing the amount of carrier material in the formulation, lipids have a lower capacity to contact between them, preventing the formation of aggregates, thus reducing the particle size of liposomes upon hydration of proliposomes. For the studied ratios, the polydispersity indexes were similar, varying from 0,318 ± 0,030 to 0.328 ± 0.014. The amount of mannitol in the formulation also seems to influence the surface charge of liposomes (**Figure 26**). Similarly to recent findings, the zeta potential becomes

insignificantly slight more negative as the weight ratio carrier: lipid increases [100]. A previous study also demonstrated that zeta potential of vesicular structures decrease when the amount of the sugars ribose and maltose increased [101]. It seems that the values of zeta potential tend to be lower when the amount of sugar in the formulation increases.

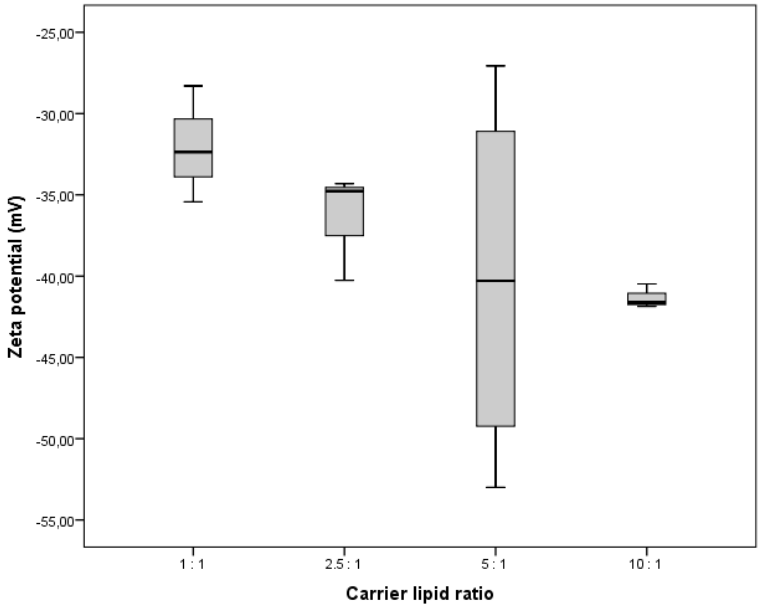


Figure 26 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by freeze drying, with different carrier : lipid weight ratio. Each box represents three individual batches.

The weight ratio between the carrier material and the lipid part 10:1 seems to form dry, free flowing powders. This was not observed for the other studied weight ratios, which produced agglomerated and not completely dried powders.

The following studies were performed with the molar ratio PC : CH (3:1) and the weight ratio carrier : lipid (10:1).

3.4. Proliposomal formulation

Maintaining the referred ratios, different percentages of **LEM2** were added to the proliposomal formulation, using film deposition on carrier and freeze drying methods. One of the studied **LEM2** percentages in the proliposomal formulations was also used to produce proliposome powders using spray drying method.

The surface morphology and thermal behavior of the obtained proliposome powders were analyzed.

3.4.1. Morphology of proliposome powders

The surface morphology of the carrier material, mannitol, and of the proliposome powders, without the drug and with **LEM2**, produced by the different methods was examined by Scanning Electron Microscopy (SEM).

For film deposition on carrier, the surface morphology of mannitol with particle size <125 μm was observed, before the preparation of proliposomes, and exhibited a porous surface (**Figure 27A and B**). The porous surface is illegible in proliposome powders, without the drug (**Figure 27C and D**) or with **LEM2** (**Figure 27E and F**), because of the deposition of phospholipids on the surface of mannitol, as described in earlier studies [35, 102]. The incorporation of **LEM2** in the proliposomal formulation (**Figure 27E and F**) does not seem to introduce major alterations to the surface morphology when compared to the proliposome powders with no drug (**Figure 27C and D**).

For the freeze drying, a solution of mannitol submitted to freeze drying in the same conditions as the solution prepared to produce proliposomes, was observed by SEM. Mannitol seems to have some orientation, similar to a crystalline structure (**Figure 28A and B**). Regarding to proliposomal powders, they seem to have heterogeneous shapes, ranging from elongated and sharped particles to approximately spherical and porous particles (**Figure 28C and D**). The presence of **LEM2** does not seem to influence the morphology at surface of freeze drying proliposome powders (**Figure 28E and F**).

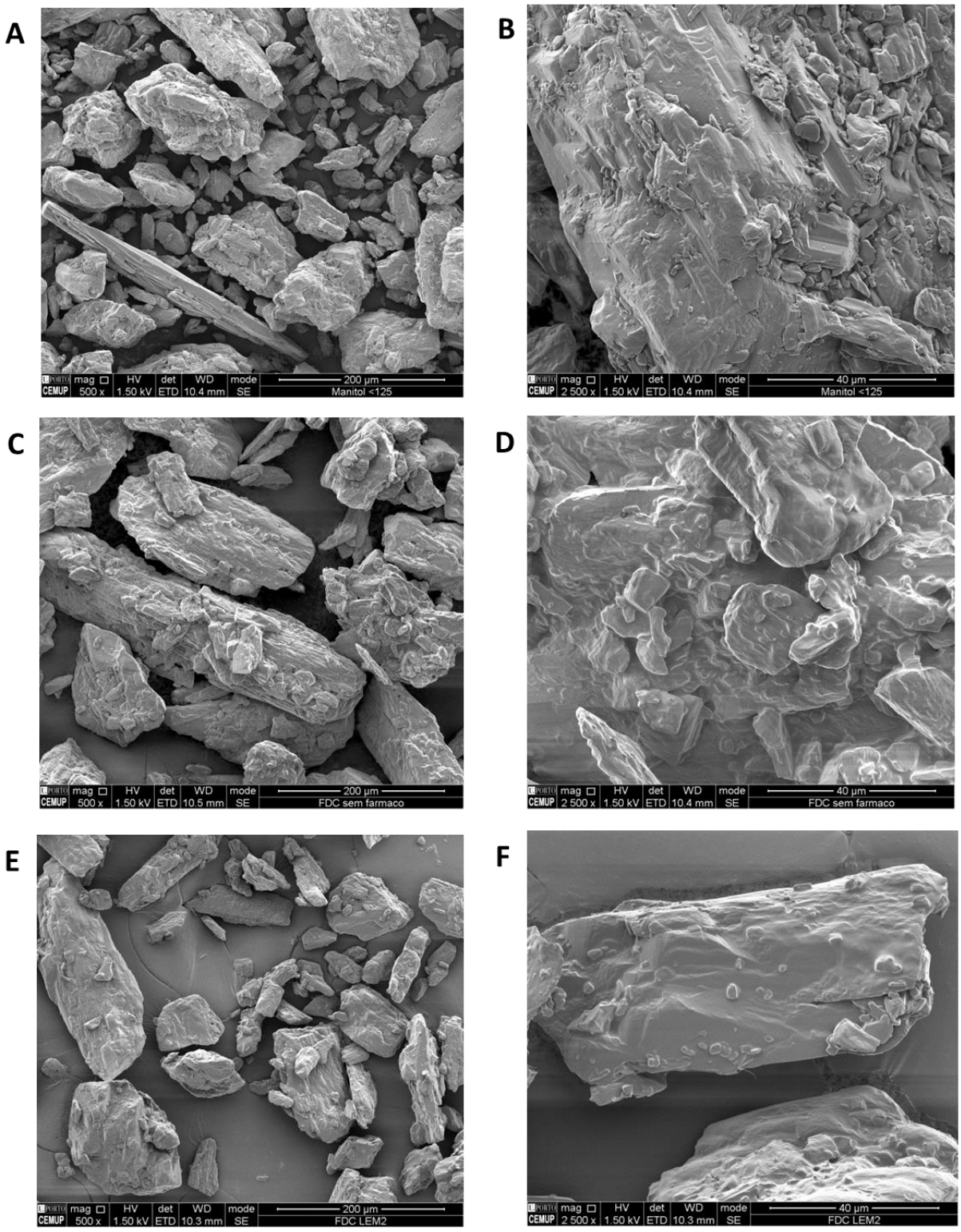


Figure 27 –SEM images of the surface of mannitol (**A-B**) and proliposome powders without drug (**C-D**) and with **LEM2** (**E-F**) produce from the film deposition on carrier method.

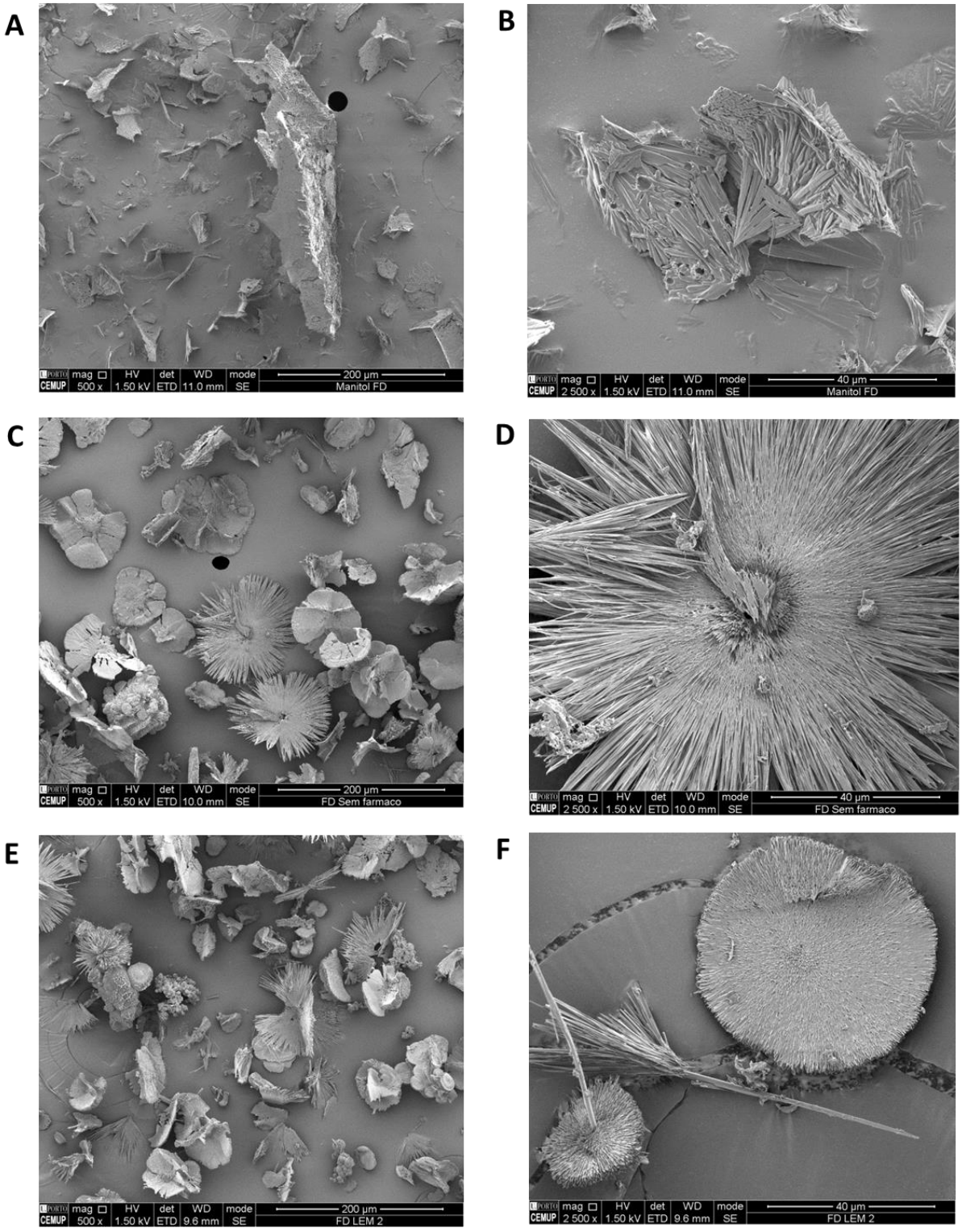


Figure 28 - SEM images of the surface of mannitol (**A-B**) and proliposome powders without drug (**C-D**) and with **LEM2** (**E-F**) produce from the freeze drying method.

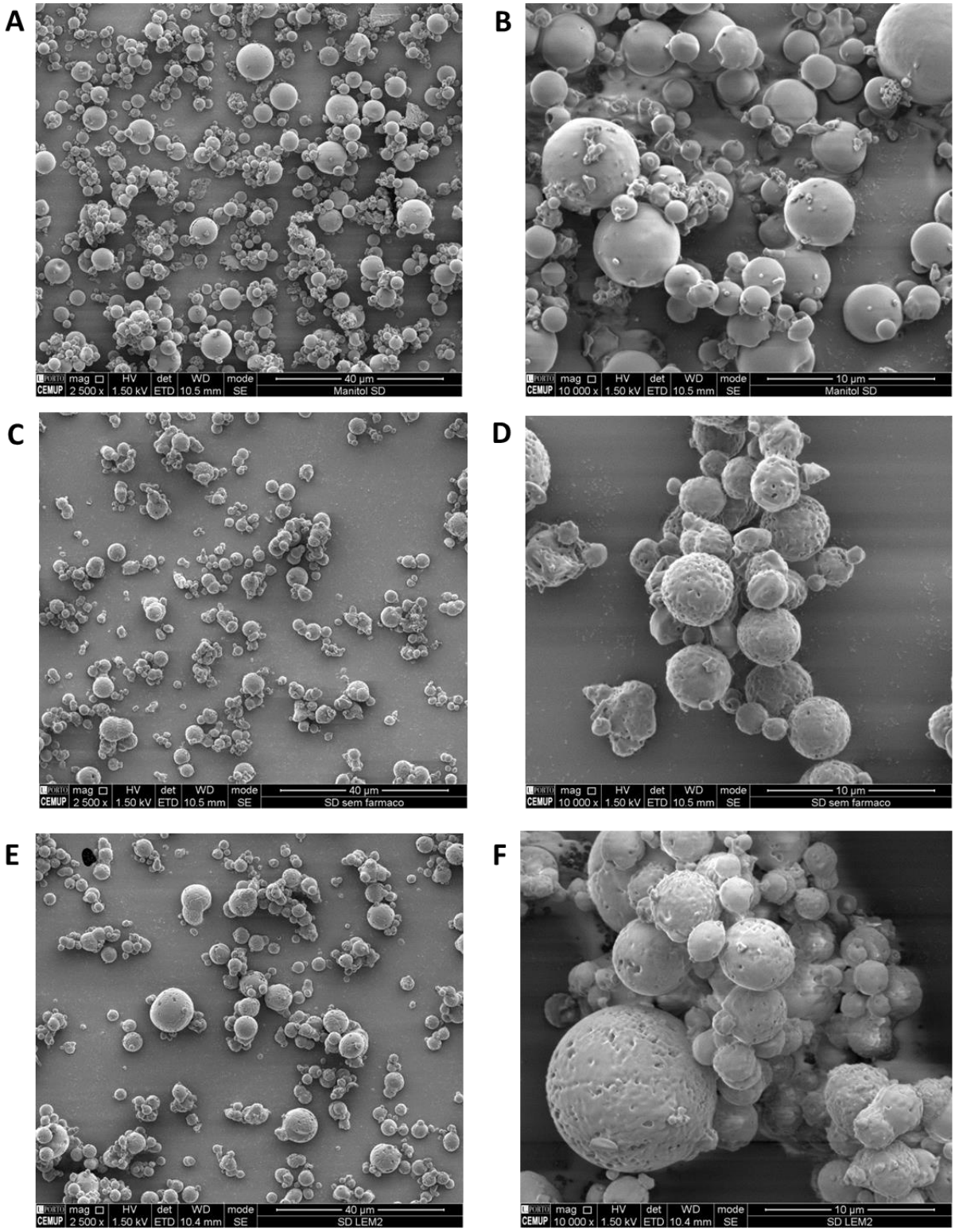


Figure 29 - SEM images of the surface of mannitol (**A-B**) and proliposome powders without drug (**C-D**) and with **LEM2** (**E-F**) produce from the spray drying method.

Figure 29 shows the morphology of spray dried mannitol particles and proliposome powders produced by spray drying. This method produces spherical particles, with a high surface area. In contrast with the film deposition on carrier proliposome powders, the spray dried mannitol exhibited a smooth surface (**Figure 29A and B**) while the proliposomal powders presented a porous surface (**Figure 29C-D**). Rojanarat *et al.* have demonstrated that mannitol has a great importance in the production of spherical particles using spray drying [38, 59-60]. If the amount of mannitol present in the formulation is not sufficient, irregular shaped particles might be obtained. As observed by the other methods, the presence of **LEM2** in spray dried proliposomes does not alter the morphology of the surface of spray drying proliposome powders (**Figure 29E and F**).

From the three methods, spray drying seems to produce more uniform proliposome powdered particles with a porous structure, which might allow a fast dispersion of the powder when hydrated to form liposomes [57]. Also, for the three methods, the incorporation of **LEM2** within proliposomes did not alter the morphology of the particles, which was already expected since the percentage of the compound in the formulation is low (2%).

3.4.2. Thermal behaviour of proliposome powders

The thermal behavior of proliposome powders was analyzed by differential scanning calorimetry (DSC), which evaluates the enthalpy variation (ΔH) and measures how the temperature changes the heat capacity of the material [103].

Figure 30 and **Table VII** present the DSC data from the components of proliposomal formulations. The mannitol peak in thermogram was used a as a reference peak for the thermal study of the proliposomal formulations, as it was done in previous studies [38, 59-60].

The DSC thermogram and DSC data from film deposition on carrier proliposomes are shown in **Figure 31** and **Tale VIII**. It was observed that proliposomal formulations present a temperature onset of approximately 2-6 ºC lower than the observed for the pure mannitol. This indicates a possible interaction of mannitol with the lipid part of the formulation, since PC and CH both present lower onset temperatures.

The presence of **LEM2** in film deposition on carrier proliposomes seems to lead to lower enthalpy values and broader peak intervals (**Figure 31** and **Tale VIII**). These alterations might be a possible indication of the interaction between **LEM2** and mannitol.

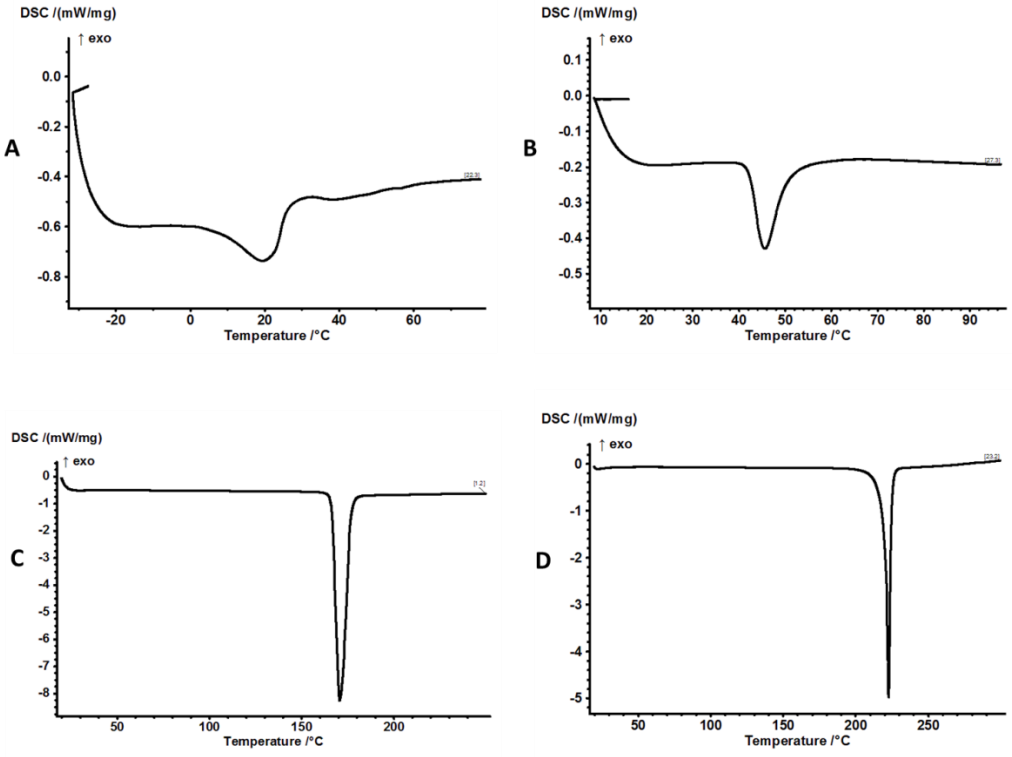


Figure 30 - DSC thermogram of egg phosphatidylcholine (**A**), cholesterol (**B**), mannitol (**C**) and **LEM2** (**D**).

Table VII – DSC data of thermograms of egg phosphatidylcholine, cholesterol, mannitol and **LEM2**.

Formulation	**Area (J/g)**	**Onset (ºC)**	**Peak (ºC)**	**End (ºC)**
PC	-12.0	7.3	19.3	25.9
CH	-7.0	42.1	45.6	50.5
Mannitol	-300.8	166.2	170.5	176.7
LEM2	-135.9	220.1	222.8	224.7

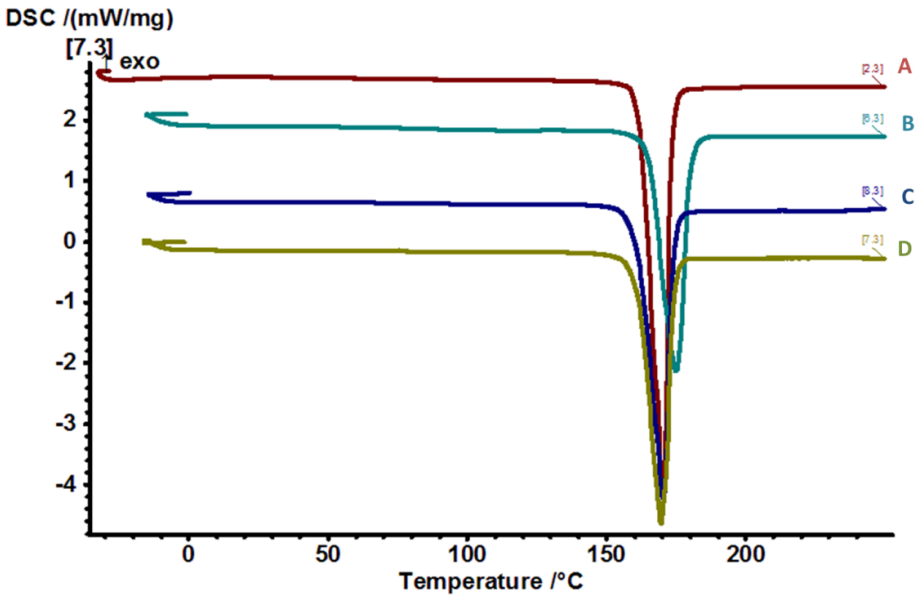


Figure 31 – DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with no drug (**A**), 0.8% **LEM2** (**B**), 2% **LEM2** (**C**) and 4% **LEM2** (**D**) obtained with film deposition on carrier method.

Table VIII –DSC data of thermograms of **LEM2**, mannitol and proliposomes with no drug, and with 0.8%, 2% and 4% of **LEM2,** obtained with film deposition on carrier method.

Formulation	**Area (J/g)**	**Onset (ºC)**	**Peak (ºC)**	**End (ºC)**
No drug	-304.8	163.8	170.2	173.3
0.8 % LEM2	-274.5	164.7	174.9	179.8
2 % LEM2	-260.3	160.4	170.4	174.1
4 % LEM2	-257.2	161.0	169.7	174.1

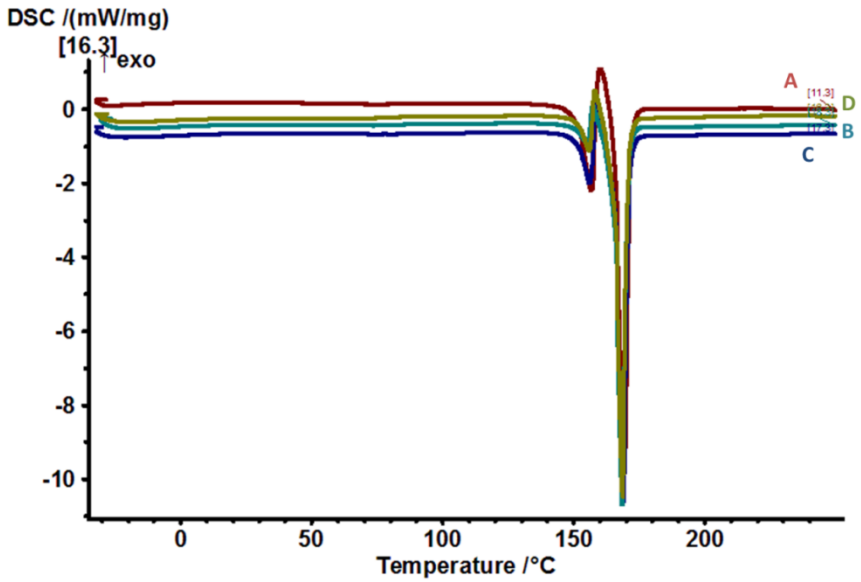


Figure 32 - DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with no drug (**A**), 0,8% **LEM2** (**B**), 2% **LEM2** (**C**) and 4% **LEM2** (**D**) obtained with freeze drying method.

Table IX – DSC data of **LEM2**, mannitol and proliposomes with no drug, and with 0,8%, 2% and 4% of **LEM2,** obtained with freeze drying method.

Formulation	Area (J/g)	Onset (ºC)	Peak (ºC)	End (ºC)
No drug	-302.5	165.7	169.2	171.2
0.8% LEM2	-262.2	165.3	168.5	170.2
2% LEM2	-268.3	165.5	168.9	170.6
4% LEM2	-263.1	165.5	168.6	170.2

Figure 32 and **Table IX** present DSC thermograms and DSC data of freeze dried proliposomes. From the thermograms it was observed the presence of two peaks, indicating that the used formulations were below the eutectic composition of the system [104].

The presence of lipids in the freeze dried proliposomes did not introduced significant alterations to the enthalpy or peak's temperature. As the observed for film deposition on

carrier proliposomes, when **LEM2** was introduced in the formulation, the enthalpy decreases, possibly due to an interaction of **LEM2** with mannitol, the carrier material.

For spray dried proliposomes the carrier material used was spray dried mannitol. In this case, the reference peak is that on the thermogram of the spray dried mannitol (**Figure 33**).

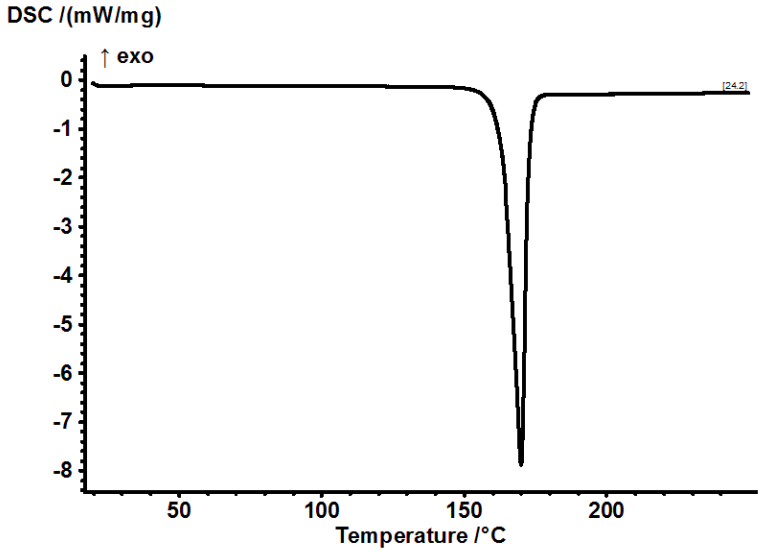


Figure 33 - DSC thermogram of spray dried mannitol from -20 ºC to 240 ºC at 10 ºC/min.

Figure 34 and **Table X** present the DSC thermograms and DSC data of spray dried proliposomes. The presence of lipids in the formulation decreases the enthalpy and the onset temperature of the peak, revealing the interaction between the lipid part and the carrier material of the formulation. The presence of **LEM2** in the formulation does not alter the onset or the end temperature of the peak. However,**LEM2** increases the enthalpy of the peak for spray dried proliposomes, as the opposite to film deposition on carrier proliposomes and freeze dried proliposomes. This might suggest that using spray dried mannitol, instead of commercial mannitol, might alter the type of interaction between **LEM2** and mannitol.

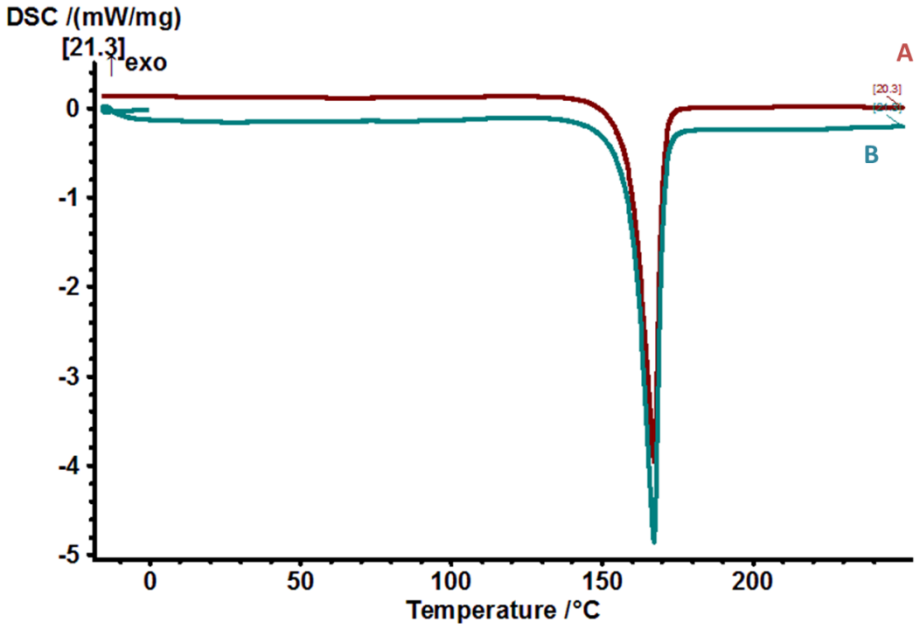


Figure 34 - DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with no drug (**A**), 2% **LEM2** (**B**) obtained with spray drying method.

Table X –DSC data of **LEM2**, spray dried mannitol and proliposomes with no drug and with 2% **LEM2** obtained with spray drying method.

Sample / Formulation	**Area (J/g)**	**Onset (ºC)**	**Peak (ºC)**	**End (ºC)**
Spray dried mannitol	-277.1	163.5	169.9	172.5
No drug	-201.3	159.6	167.2	170.1
2% LEM2	-240.4	159.6	167.2	170.2

3.5. Hydration of proliposomes

Membrane lipids tend to form vesicles when in contact with water [27, 33]. Thus, the proliposome powders were hydrated using miliQ water and agitated to obtain liposomal dispersions, and then filtered through a 5 μm filter. Liposomes were characterized regarding to surface morphology, size of particles, surface charge and entrapment efficiency of **LEM2**.

3.5.1. Morphology of liposome dispersions

Figure 35 shows the surface morphology of the particles obtained by hydration of proliposomes produced by film deposition on carrier. It was observed the formation of particles having a spherical shape, which are attributed to liposomal vesicles. However, particles with a not completely spherical structure could also be observed (**Figure 35A and C)**. Besides, the hydration of proliposomes can also lead to formation of an uncommon type of structure (**Figure 35B and D**), which seems to be a vacuole-like structure, with a diameter of 4-5 μm and comprising in its interior a number of irregular particles at the nanometer scale. The diameter of liposomes, either without drug or with **LEM2**, ranges from hundreds of nanometers to a few micrometers (**Figure 35A and C**).

The morphology of the particles formed by hydration of freeze dried proliposomes is represented in **Figure 36**. It was observed that the hydration leads to the formation of particles with a more uniform shape than the observed for the film deposition on carrier method. Spherical particles from hundreds of nanometers to a few micrometers were observed by cryoSEM images (**Figure 36**). The vacuole-like structures were also observed for liposomes generated from freeze dried proliposomes (**Figure 36D**). However, they seem to be present in a lower extent than in the film deposition on carrier method and with smaller sizes (around 2 μm).

The hydration of the spray dried proliposomes originated spherical particles (**Figure 37**), with a uniform shape in the nanometer scale. The vacuole-like structures were present in cryoSEM images (**Figure 37B**) at a low extent with a diameter of about 2 μm.

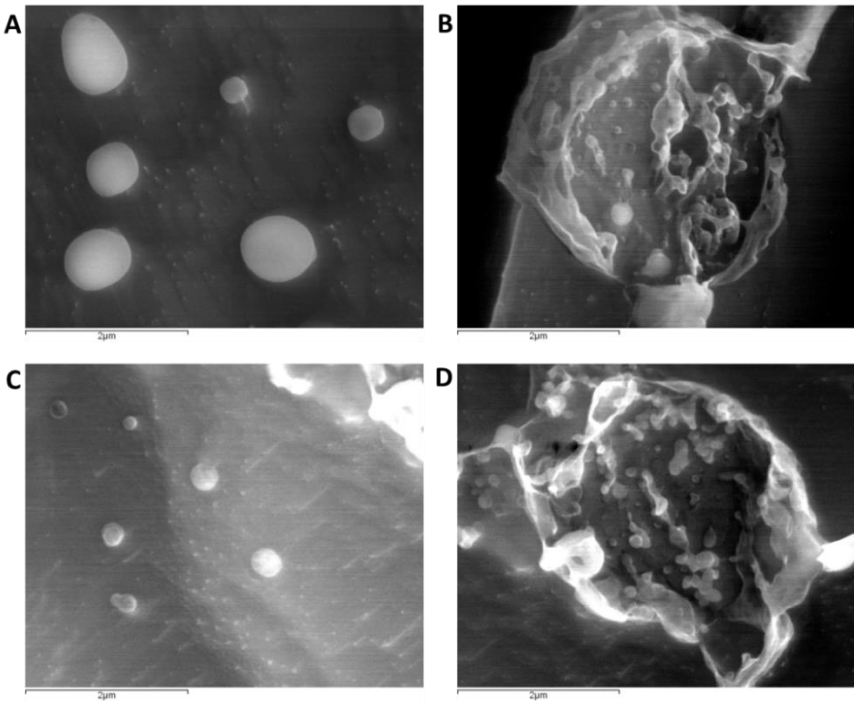


Figure 35 - CryoSEM images of liposomes formed by hydration of proliposomes produced by the film deposition on carrier method, without drug (**A-B**) and with 2% of **LEM2** (**C-D**) at x 25000 magnification.

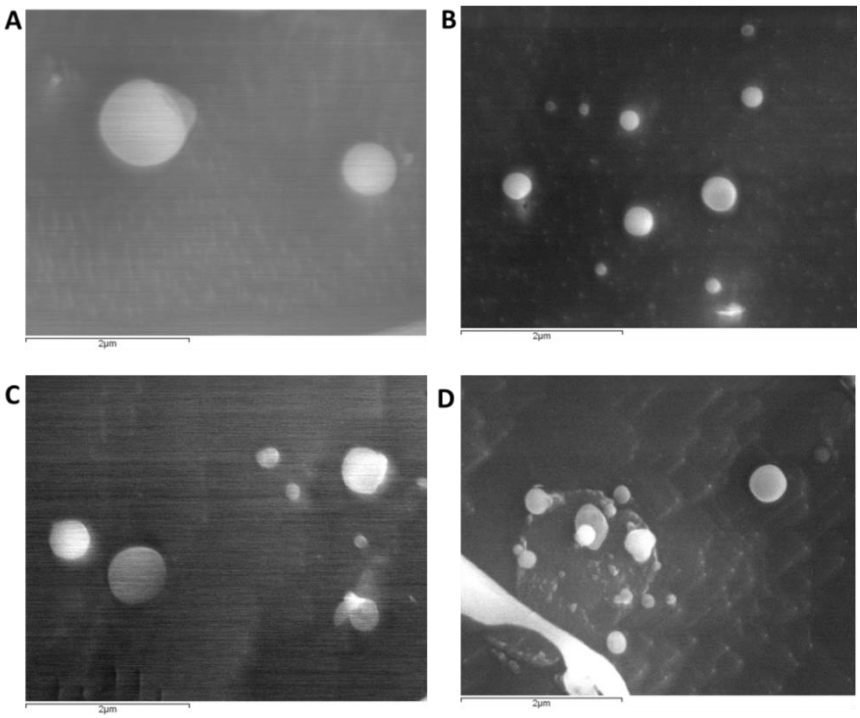


Figure 36 - CryoSEM images of liposomes formed by hydration of proliposomes produced by the freeze drying method, without drug (**A-B**) and with 2% of **LEM2** (**C-D**) at x 25000 magnification.

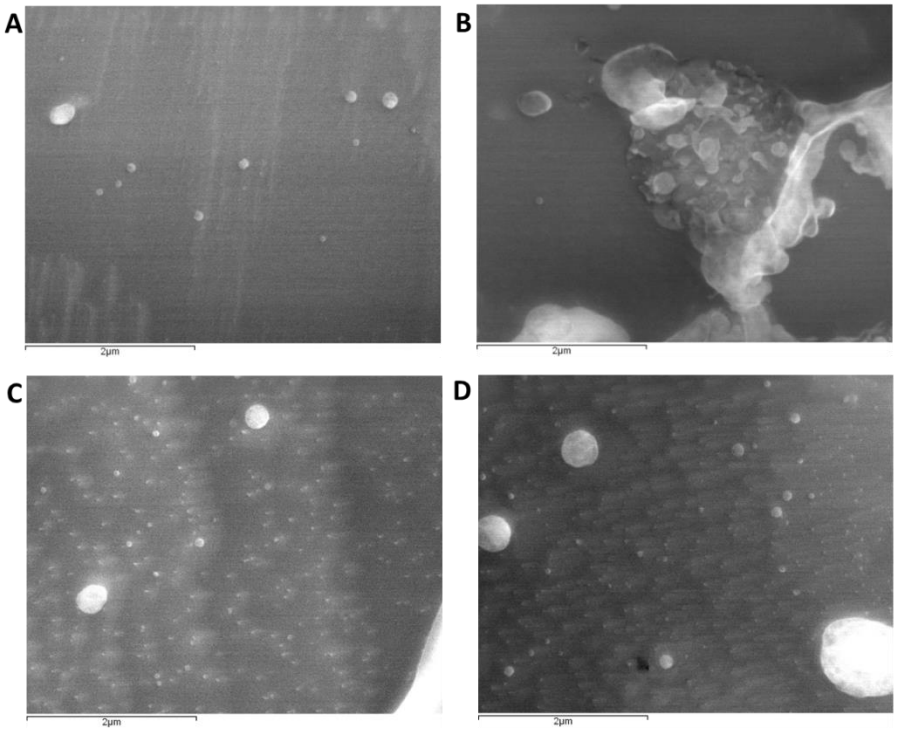


Figure 37 - CryoSEM images of liposomes formed by hydration of proliposomes produced by the spray drying method, without drug (**A-B**) and with 2% of **LEM2** (**C-D**) at x 25000 magnification.

Liposomes obtained by hydration of spray dried proliposomes presented a smaller size than liposomes formed by hydration of proliposomes produced by both film deposition on carrier and freeze drying methods.

The vacuole-like structures were observed for the three analyzed methods, with a higher extent for the film deposition on carrier method. For the freeze drying and spray drying methods, these structures were less evident and presented smaller diameters.

3.5.2. Particle size

The effective diameters presented in this work were evaluated by Dynamic Light Scattering (DLS), using an apparatus which is suitable to measure the diameter of particles until 5 µm. Thus, particles with a higher diameter were not counted for the medium values of effective diameter. However, after the hydration of proliposomal formulations, the dispersion was

filtered through a 5 μm filter. Considering this, the range of measured diameters seems to be appropriated to measure the obtained liposomes.

Figure 38 presents the intervals and the medium effective diameter of liposomes formed by hydration of film deposition on carrier proliposomes. The medium diameter seems to insignificantly increase with the presence of **LEM2** comparing to the formulation with no drug (546.5 ± 66.0 nm). The effective diameter of liposomes with **LEM2** ranged from 592.3 ± 42.6 nm to 659.1 ± 152.37 nm, and it was observed that increasing the **LEM2** percentage in the formulation, higher effective diameter values were obtained. However, this difference is not statistically significant.

The effective diameter of liposomes generated by hydration of freeze dried proliposomes is shown in **Figure 39**. The formulation with no drug has a medium effective diameter of 443.60 ± 26.80 nm and for the formulations with drug, the medium effective diameter values range from 410.83 ± 17.24 nm to 450.63 ± 37.95 nm. Surprisingly, the effective diameter insignificantly decreased when the **LEM2** percentage was increased.

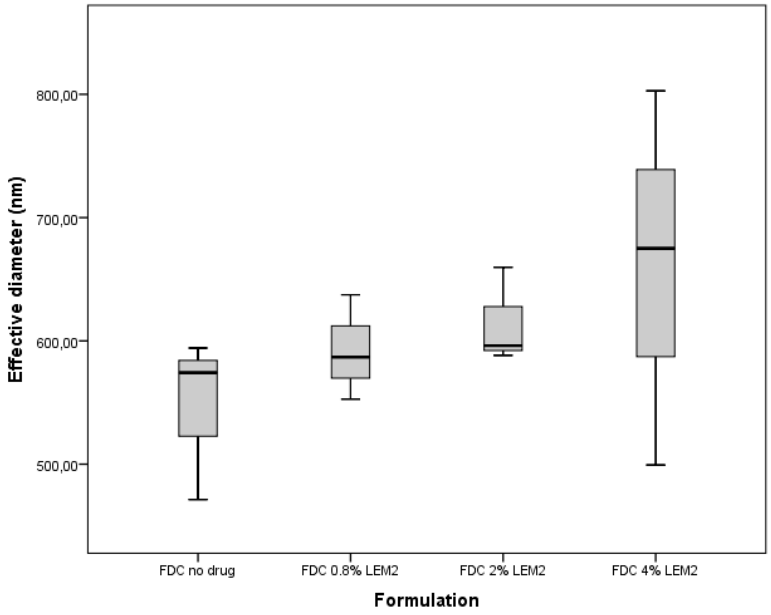


Figure 38 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

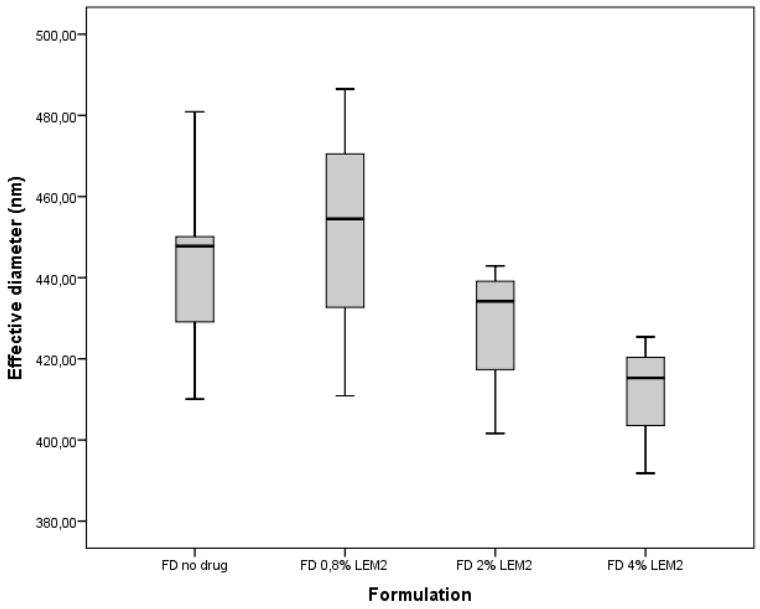


Figure 39 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by freeze drying, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

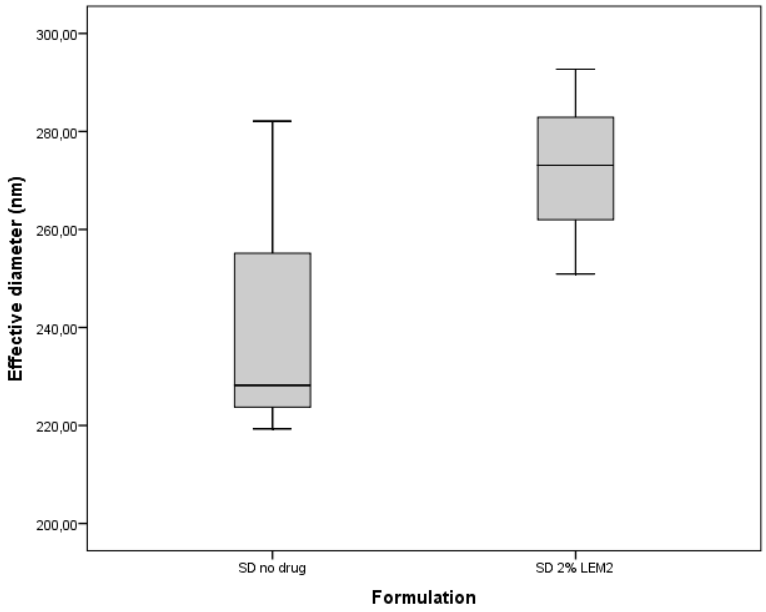


Figure 40 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by spray drying, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

The effective diameter of liposomes formed from hydration of spray dried proliposomes is present in **Figure 40**. The formulation with no drug has a medium effective diameter of 243,2 ± 34,0 nm and the formulation with 2% of **LEM2** has a higher effective diameter, of 272,2 ± 20,9 nm. This difference has no statistical significance.

From the three studied methods, the effective diameter increases in the following order spray drying < freeze drying < film deposition on carrier.

The polydispersity indexes of the presented formulations ranged between 0,272 ± 0,024 and 0,353 ± 0,019 (**Appendix II**), with the spray drying method presenting the lower values. This indicates that all the formulations were polydispersed, which agrees with the cryoSEM analysis presented above. The cryoSEM images revealed the presence of particles with a wide range of sizes.

3.5.3. Zeta potential

Zeta potential is a physical property that allows the indirect measurement of the surface charge. The magnitude of this property gives an idea about long-term stability of the colloidal dispersion. If zeta potential of liposomes in dispersion has a high absolute value, there is a tendency for repulsion between the nanoparticles, avoiding flocculation or coagulation. Thus, zeta potential value must be more positive than + 30mV or more negative than – 30mV, in order to be considered stable by electrostatic repulsion [105].

For all the studied formulations, zeta potential was more negative than -30 mV, indicating electrostatic stability (**Figure 41-43**).

For all the studied methods, the zeta potential became less negative when **LEM2** was introduced in the formulation. For the formulations with no drug, zeta potential was between -41.84 ± 2.66 mV and -47.38 ± 8.19 mV, while for the formulations with **LEM2** the zeta potential values ranged from -37.83 ± 1.49 and -38.63 ± 0.40 mV. These differences did not present significant statistical meaning. Besides, between the different **LEM2** percentages, the zeta potential values were similar (**Figure 41-42**).

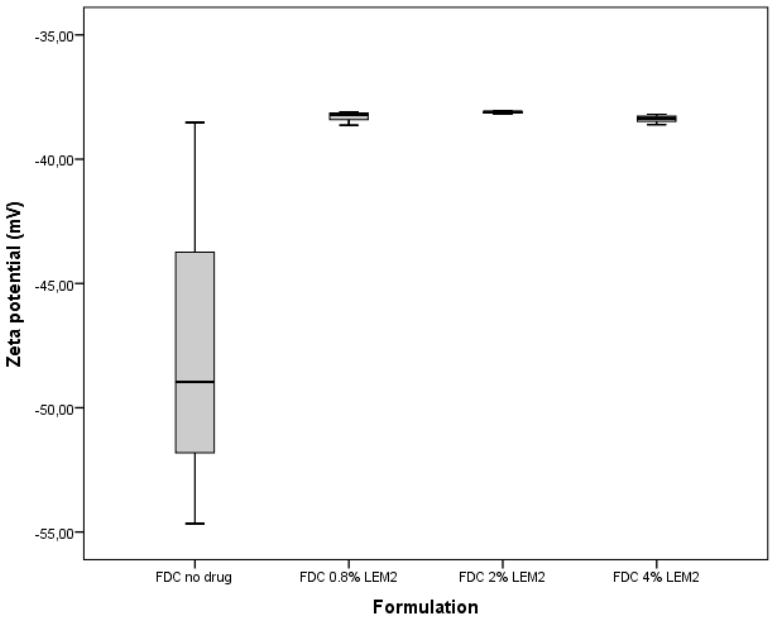


Figure 41 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

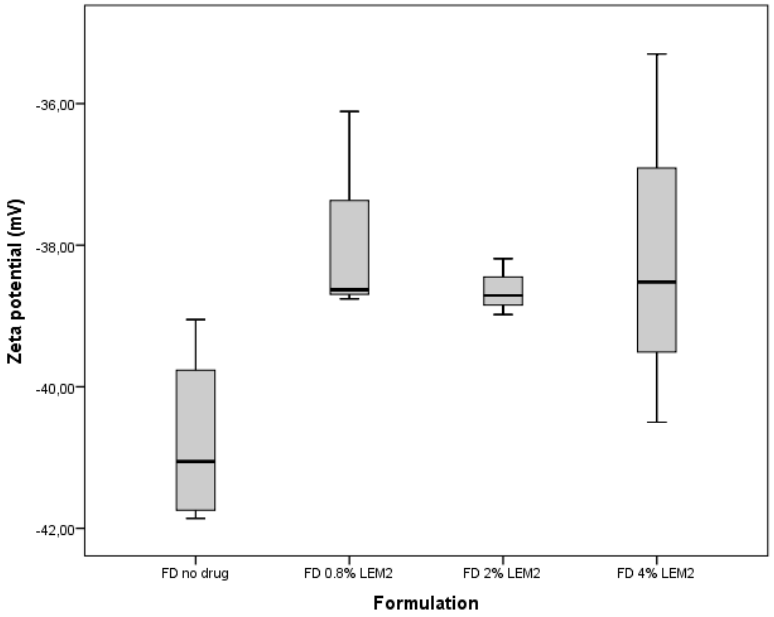


Figure 42 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by freeze drying, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

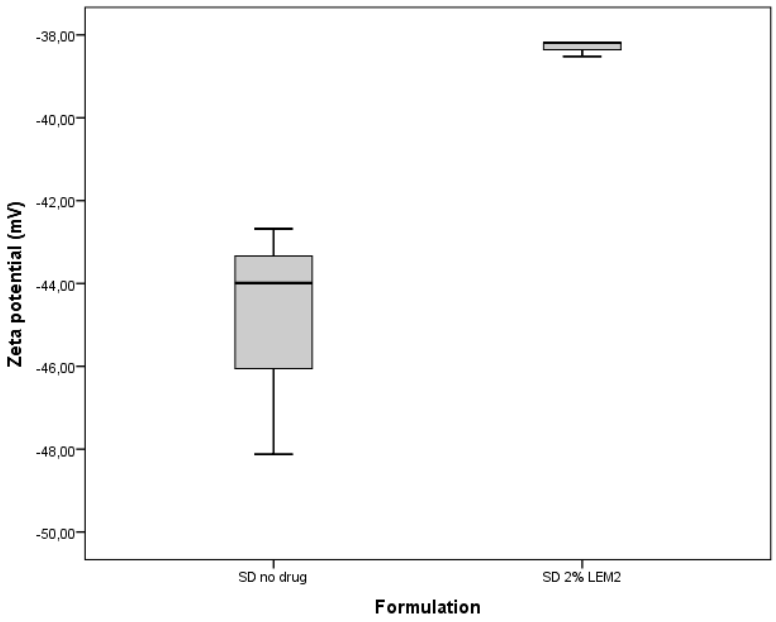


Figure 43 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by spray drying, with no drug and with different percentages of **LEM2**. Each box represents three individual batches.

3.5.4. Entrapment efficiency

The entrapment efficiency of **LEM2** in liposomes obtained by hydration of proliposomes was determined by a direct measurement of the compound that was encapsulated in the formulation. The quantification of **LEM2** was performed by an HPLC method.

For the film deposition on carrier and freeze drying methods, three percentages of **LEM2** were studied: 0.8%, 2% and 4%. **Figures 44** and **45** demonstrate that the higher entrapment efficiency was reached for 2% of **LEM2** in the formulation and that at 4% of **LEM2**, the entrapment efficiency was even lower than at 0.8% of **LEM2**. Apparently, liposomes have a limited capacity to entrap **LEM2**, which is maximal at 2% of **LEM2** for the total weight of the formulation.

For the freeze drying method, the entrapment efficiency at 2% of **LEM2** (62.68 ± 31.55 %) was significantly higher than at 4% of **LEM2** (4.72 ± 0.55%) ($p < 0.05$). However,

other differences observed in the entrapment efficiency between formulations were not statistically relevant.

Since the entrapment efficiency of liposomes for **LEM2** was higher at 2% of the compound on the total weight of the proliposomal formulation, this was the chosen percentage to produce spray dried proliposomes (**Figure 46**).

From the three studied methods, at 2% of **LEM2**, the entrapment efficiency increases in the following order: spray drying (47.54 ± 24.73%) < film deposition on carrier (51.92 ± 21.91%) < < freeze drying (62.68 ± 31.55%).

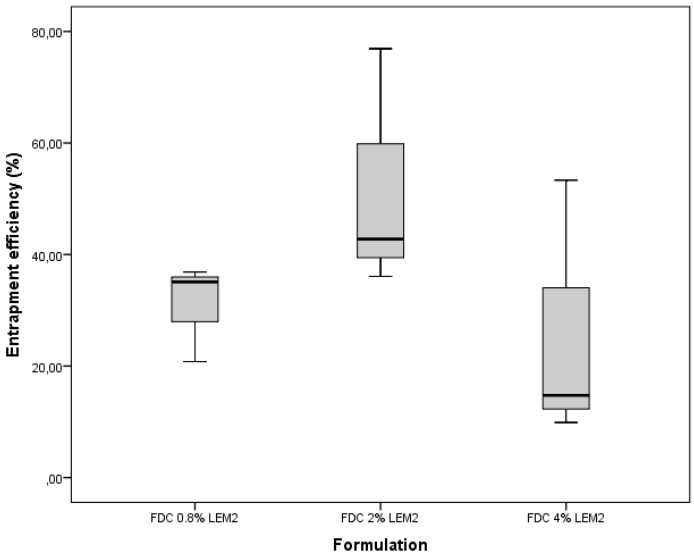


Figure 44 – Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with different percentages of **LEM2**. Each box represents three individual batches.

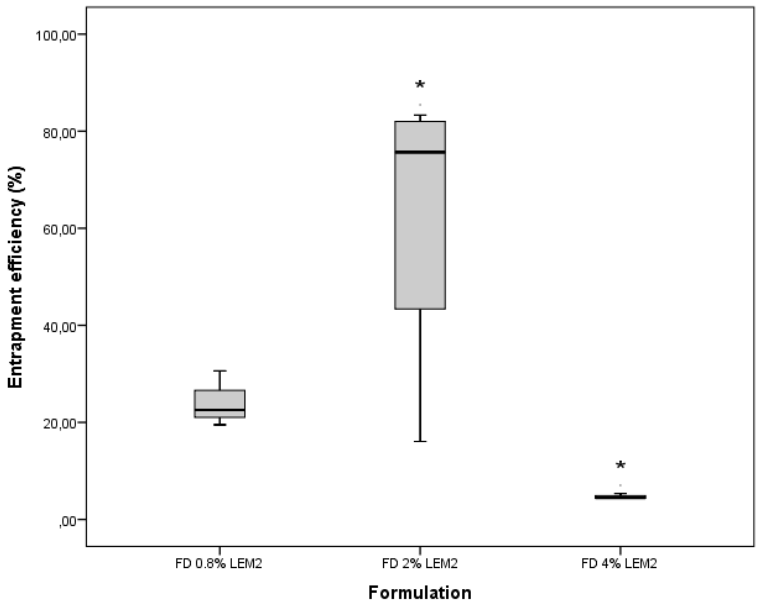


Figure 45 – Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by freeze drying, with different percentages of **LEM2**. Each box represents three individual batches. Statistical significance: * $p = 0.02$.

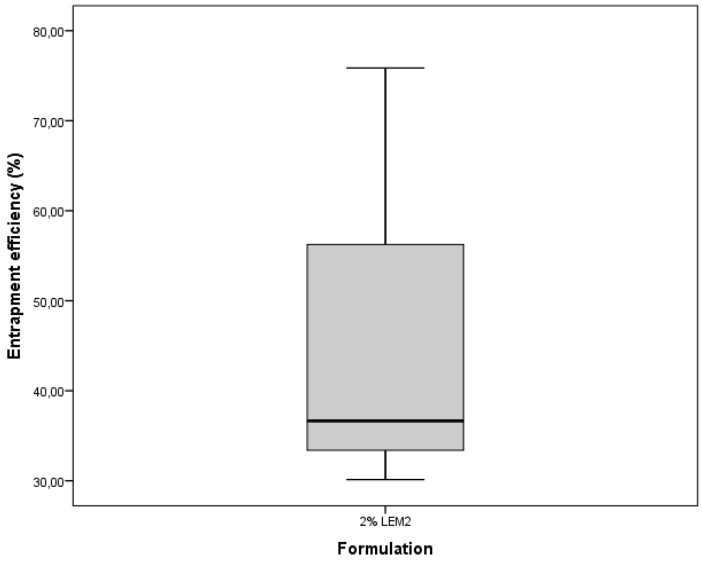


Figure 46 – Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by spray drying, with 2% of **LEM2**. Each box represents three individual batches.

3.6. Stability of proliposomes

After producing proliposomes, they were kept in a desiccator at room temperature. The stability studies were performed up to 30 days. Proliposomes were analyzed by DSC and hydrated to characterize the obtained liposomes.

3.6.1. Thermal behavior of proliposome powders

The DSC thermograms of proliposomes, 30 days after their production exhibited overlapping peaks, whit slight alterations comparing to the day of production.

For film deposition on carrier proliposomes (**Figure 47** and **Table XI**), it seems that 30 days after their production, the peak suffer a small shift to higher temperatures and the enthalpy was increased.

For freeze dried proliposomes, the major alteration observed after 30 days of the production is the decrease in the enthalpy of the peak (**Figure 48** and **Table XII**).

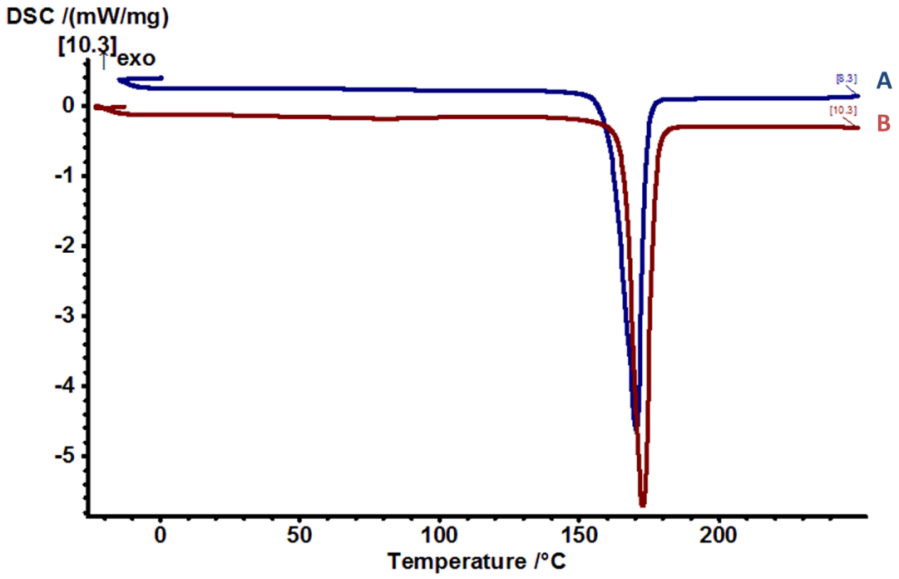


Figure 47 - DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with 2% of **LEM2** at the day of production (**A**) and at day 30 (**B**) obtained with film deposition on carrier method.

Table XI – DSC data of proliposomes with 2% of **LEM2** at the day of production and at day 30, obtained with film deposition on carrier method.

Day	Area (J/g)	Onset (ºC)	Peak (ºC)	End (ºC)
0	-260.3	160.4	170.4	174.1
30	-267.1	165.8	172.8	177.0

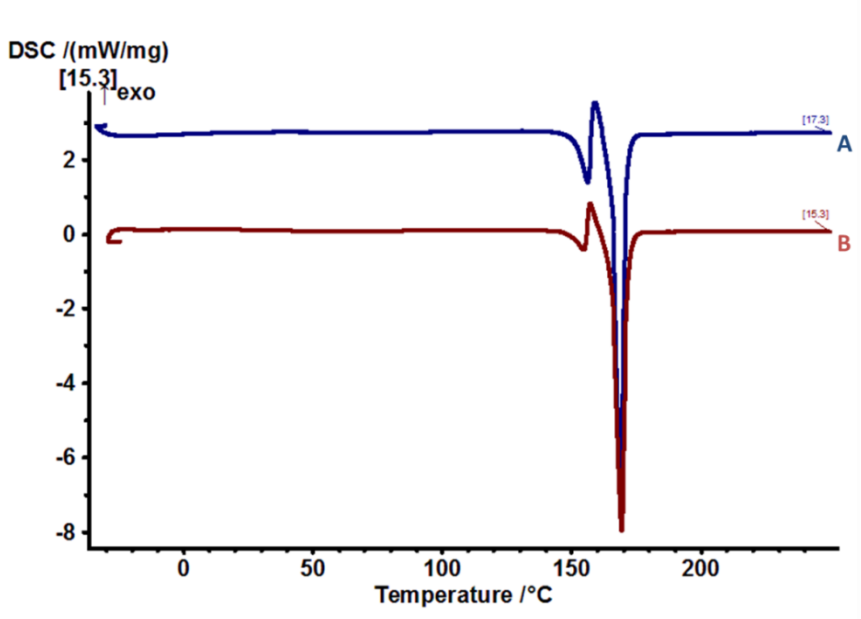


Figure 48 - DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with 2% of **LEM2** at the day of production (**A**) and at day 30 (**B**) obtained with freeze drying method.

Table XII – DSC data of proliposomes with 2% of **LEM2** at the day of production and at day 30, obtained with freeze drying method.

Day	Area (J/g)	Onset (ºC)	Peak (ºC)	End (ºC)
0	-268.3	165.5	168.9	170.6
30	-228.1	165.3	169.2	171.3

Spray dried proliposomes, 30 days after the production exhibited a lower enthalpy, as observed for freeze dried proliposomes. Also, the onset temperature of the peak decreased and the peak became narrower (**Figure 49** and **Table XIII**).

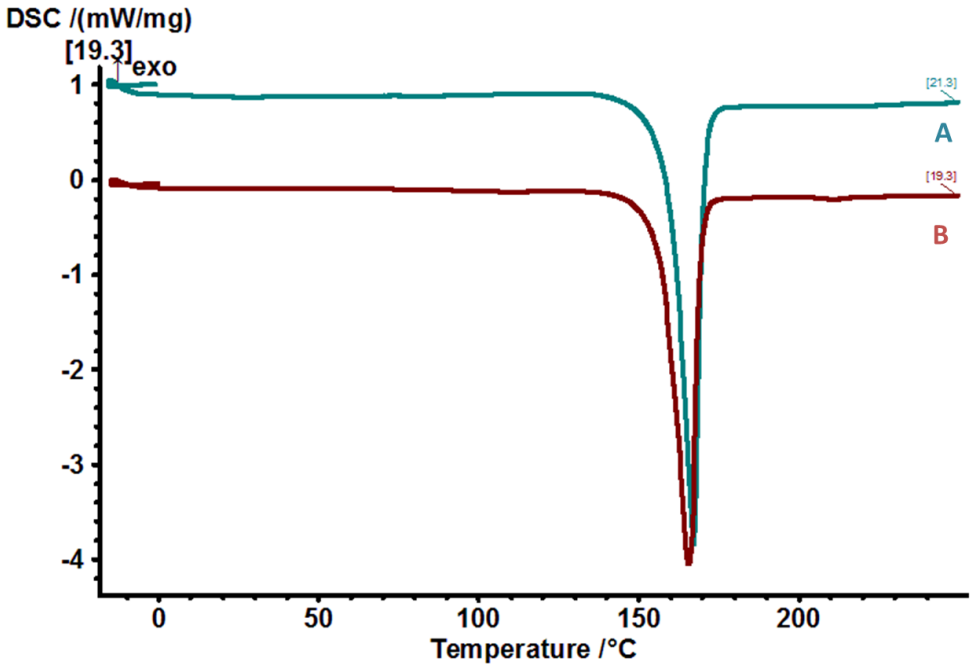


Figure 49 - DSC thermograms from -20 ºC to 240 ºC at 10 ºC/min of proliposomes with 2% of **LEM2** at the day of production (**A**) and at day 30 (**B**) obtained with spray drying method.

Table XIII – DSC data of proliposomes with 2% of **LEM2** at the day of production and at day 30 obtained with spray drying method.

Day	Area (J/g)	Onset (ºC)	Peak (ºC)	End (ºC)
0	-240.4	159.6	167.2	170.2
30	-219.5	157.5	165.5	169.2

3.6.2. Characterization of liposomes

3.6.2.1. Particle size

Figures 50 to **52** present the variation on the size of liposomes, 30 days after of the production of proliposomes. For the film deposition on carrier (**Figure 50**) and spray drying method (**Figure 52**), no statistically significant alterations were found in the effective diameter after 30 days of the production of proliposomes.

By contrast, the effective diameter of liposomes hydrated from freeze dried proliposomes significantly increased 30 days after proliposome production ($p < 0.05$) (**Figure 51**). This indicates physical instability of liposomes when using freeze drying to produce proliposomes, probably due to the formation of lipid aggregates. However, film deposition on carrier and spray drying proliposomes seem to prevent the physical instability.

At the day 30, the polydispersity indexes were similar to those measured at the day of production, for three methods (**Appendix III**).

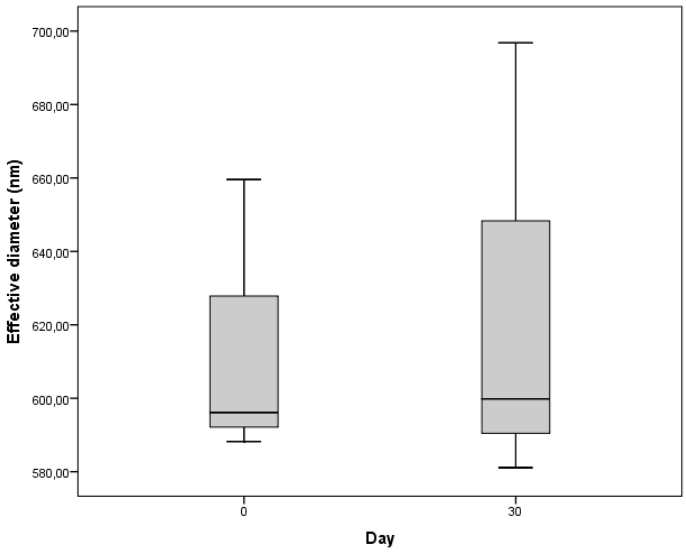


Figure 50 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

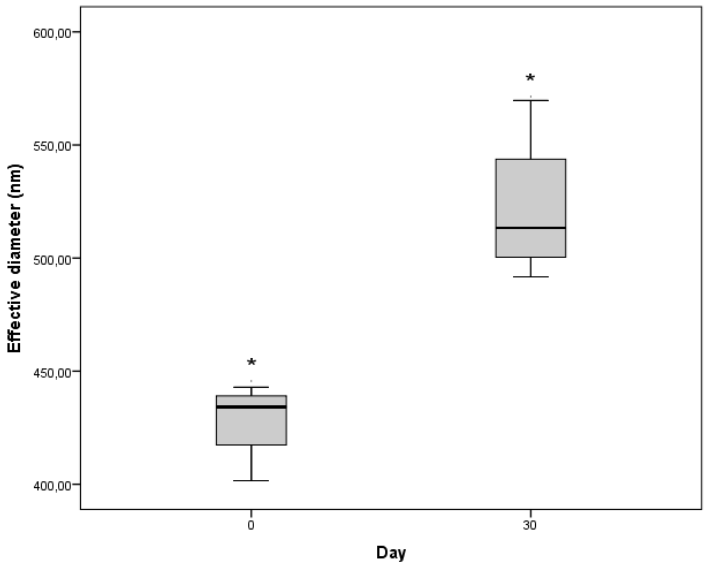


Figure 51 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by freeze drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches. Statistical significance: * $p = 0.005$.

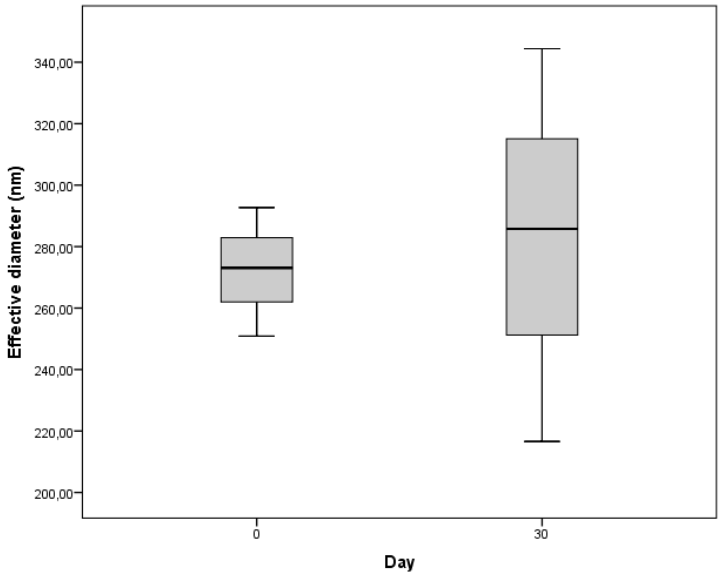


Figure 52 – Box and whiskers plot of effective diameter of liposomes formed by hydration of proliposomes produced by spray drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

3.6.2.2. Zeta potential

The zeta potential of liposomes, 30 days after the production of liposomes is shown in **Figures 53** to **55**. Liposomes hydrated from film deposition on carrier proliposomes (**Figure 53**) presented a significantly more negative zeta potential ($p < 0.05$).

For the other studied methods, no significant differences in the surface charge of liposomes were observed (**Figure 54** and **55**).

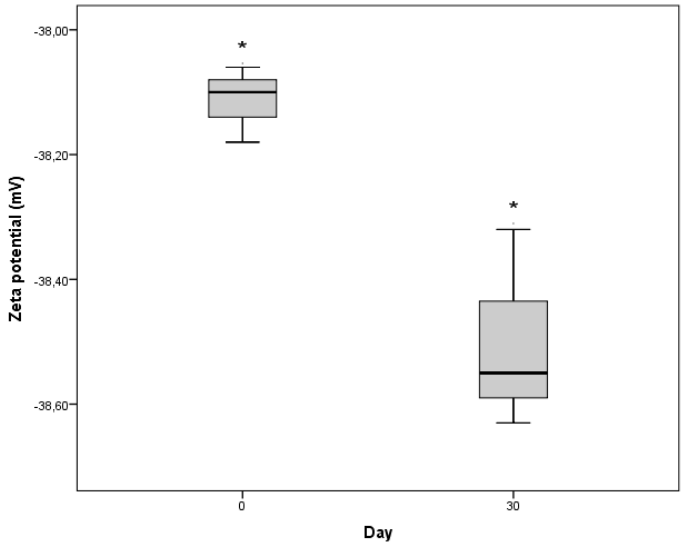


Figure 53 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches. Statistical significance: * $p = 0.018$.

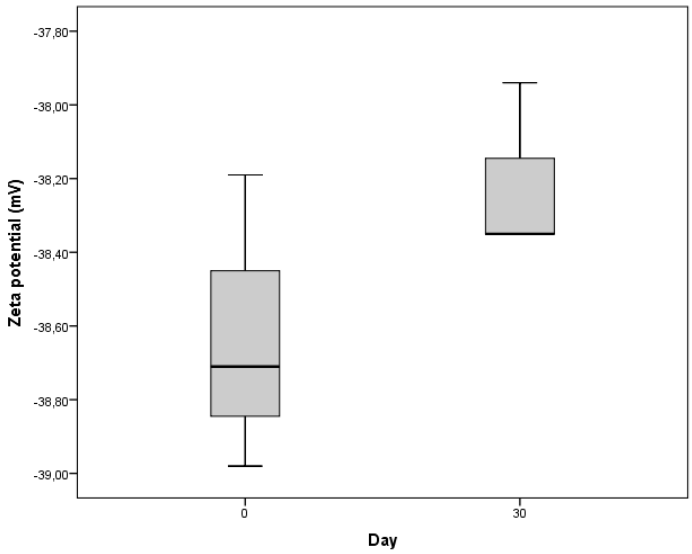


Figure 54 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by freeze drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

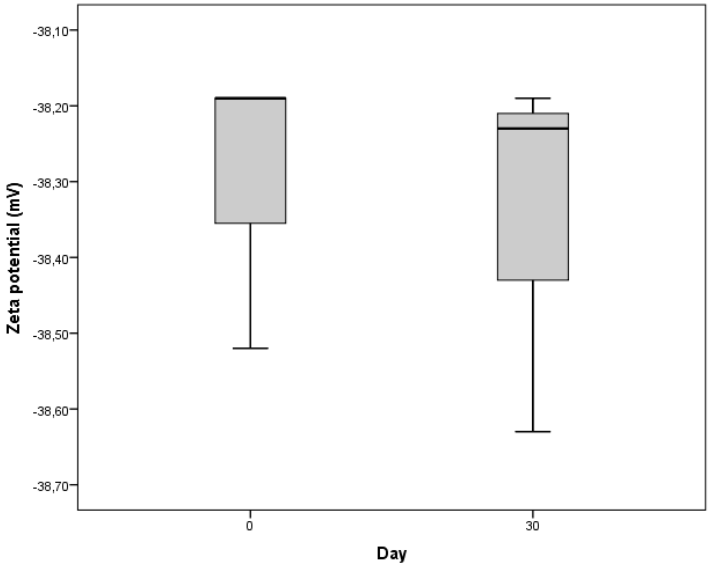


Figure 55 – Box and whiskers plot of zeta potential of liposomes formed by hydration of proliposomes produced by spray drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

3.6.2.3. Entrapment efficiency

Figures 56 to **58** present the entrapment efficiency of the hydrated proliposomes 30 days after the production of proliposomes. Although the three methods presented lower entrapment efficiencies at day 30, none of those alterations presented statistically significance. When the processes of oxidation and hydrolysis of phospholipids take place, they lead to chemical instability on liposomes, increasing the permeability of liposomes [10]. Thus, the absence of significant changes in the entrapped drug on liposomes might be an indicator that the instability of liposomes may be prevented by the use of proliposomes.

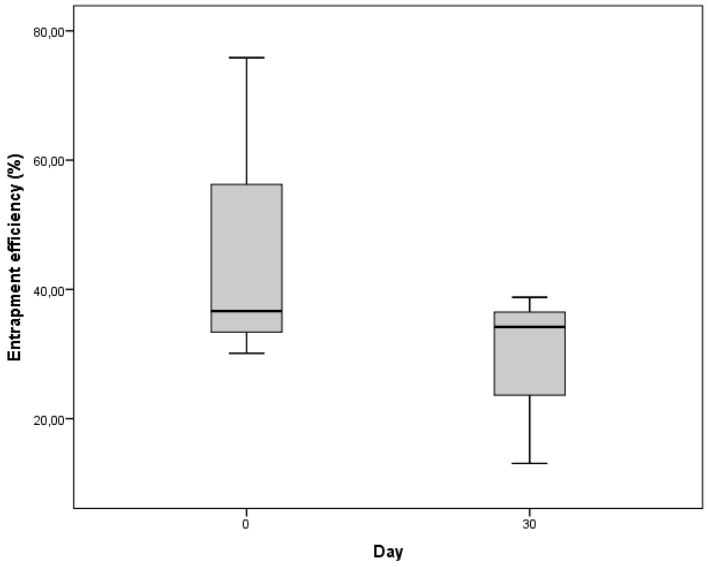


Figure 56 – Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by film deposition on carrier, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

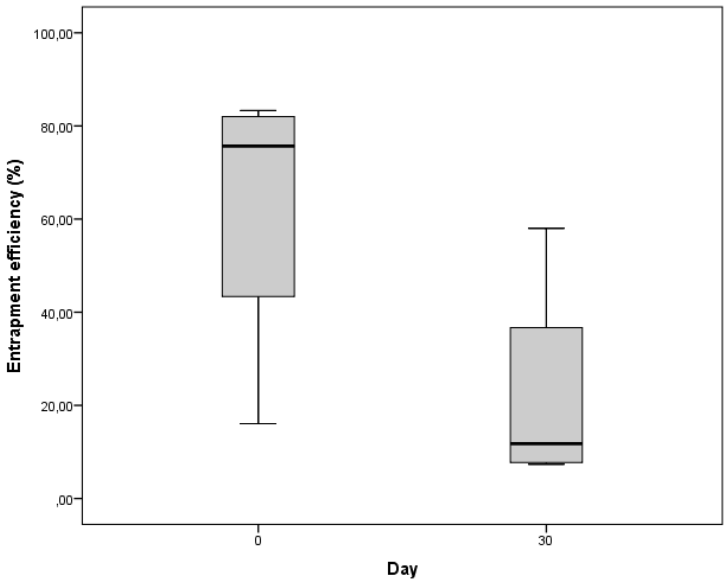


Figure 57 - Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by freeze drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

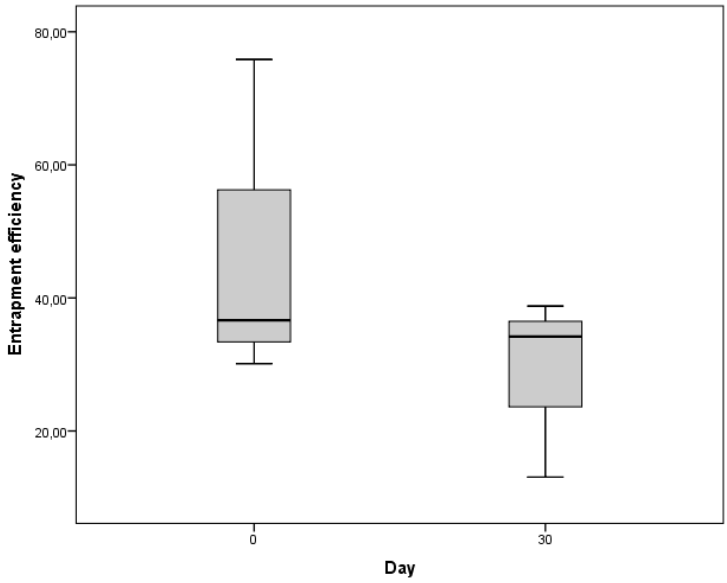


Figure 58 – Box and whiskers plot of entrapment efficiency of liposomes formed by hydration of proliposomes produced by spray drying, with 2% of **LEM2,** at the day of production and at day 30. Each box represents three individual batches.

From the characterization of liposomes obtained from proliposomes 30 days after their production, it seems that spray drying presents a better stability when compared to film deposition on carrier and freeze drying methods. 30 days after the production of proliposomes, film deposition on carrier powders presented significant alterations in zeta potential and freeze drying lead to a significant increase in the effective diameter of liposomes, while no significant alterations were observed for spray dried proliposomes.

Proliposomes were kept at room temperature for 30 days. Their storage at lower temperature would probably improve their stability. Janga *et al*. reported the production of stable film deposition on carrier proliposomes for 90 days, under refrigerated temperatures (4 ± 2 ^{o}C) [102].

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

CHAPTER 4 - CONCLUSIONS AND FUTURE WORK

Liposomes are prone to different levels of instability, restricting their shelf-life and their clinical application. Proliposomes have been studied as an alternative approach in an attempt to overcome stability problems of liposomes.

Among the different methods to produce proliposomes, film deposition on carrier, freeze drying and spray drying were used in this dissertation to incorporate the synthetic xanthone derivative **LEM2,** with a promising antitumor effect. Xanthone usually present poor solubility and their encapsulation in nanosystems might be a strategy to solve the solubility problem.

LEM2 was effectively synthesized according to a previous protocol, with a good yield. The three methods used to prepare proliposomes could effectively encapsulate **LEM2**. Each method produced a powder with different properties and with distinct surface morphologies. Spray drying method produced powders having a more uniform surface morphology, with spherical particles.

The hydration of proliposomal powders was effective in converting them in liposomes, which presented medium effective diameters in the nanometer scale and good zeta potential values. Different percentages of **LEM2** in the formulation were studied and the maximum entrapment efficiency occurs when **LEM2** is present at 2% of the total formulation. The entrapment efficiency ranged from about 48% to 63%, for that **LEM2** percentage in the formulation.

The stability of proliposomes was also accessed and spray drying presented the better stability profile, with no significant alterations in the properties of liposome, 30 days after the production of proliposomes. Film deposition on carrier showed significant altered zeta potential after 30 days of production and freeze drying presented significant alterations in the effective diameter of liposomes, 30 days after the production.

From the studied methods, spray drying seems to be most promising for the manufacture of proliposome formulations. Although spray drying presented the lower entrapment efficiency for **LEM2,** this method produces stable proliposomes with a uniform morphology.

Although spray dried proliposomes have shown to be stable 30 days after their production, further studies should be carried out to infer their stability for a prolonged period of time. Also, the stability of proliposomes should be investigated at different conditions,

mainly at different temperatures for the three production methods. The instability presented by film deposition on carrier and freeze dried proliposomes might be solved by the alteration of their storage condition.

CHAPTER 5

MATERIAL AND METHODS

CHAPTER 5 - MATERIAL AND METHODS

5.1. General Methods

Lipoid E80 (egg phospholipids with 80% of phosphatidylcholine) was a gift from Lipoid, cholesterol was purchased from Acofarma and methanol used in HPLC was purchased from VWR chemicals, with HPLC grade. Mannitol and all the other reagents and solvents were purchased from Sigma Aldrich, and had no further purification process. Solvents were evaporated using rotary evaporator under reduced pressure, Buchi Waterchath B-480. All reactions were monitored by TLC carried out on precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF254). The UV light at 254 and 365 nm was used for visualization of chromatograms. Microwave (MW) reactions were performed in glassware open vessel reactors in a MicroSYNTH 1600 Microwave Labstation from Millestone (ThermoUnicam, Portugal). The internal reaction temperature was controlled by a fiber-optic probe sensor. Purification of the synthesized compounds was performed by chromatography flash column using Merck silica gel 60 (0.040-0.063 mm), chromatography flash cartridge (GraceResolv®, Grace Company, Deerfield, IL, USA). The purity of each compound was qualitatively accessed by TLC using two different chromatographic conditions. The ultrapure water was purified by the Direct-Q® purification system of Millipore.

5.2. Synthesis of the carbaldehydic xanthone derivative LEM2

5.2.1. Synthesis of benzophenone intermediate 3, (2-hydroxy-3,4-dimethoxy-6-methylphenyl) (methoxyphenyl) methanone

$AlCl_3$ anhydrous (18.3 g, 137.2 mmol) was added to a dry ether solution (200 ml) of 2-3,4,5-trimethoxytoluene (**1,** 10 g, 54.88 mmol) and methoxybenzoyl chloride (**2,** 11.2 g, 65.85

mmol). The resulting deep red mixture was stirred at room temperature, overnight, under N_2. The reaction was monitored by TLC using *n*-hexane/ethyl acetate in a proportion of 7:3, using the reagents as control. After completing the reaction, the suspension was acidified with HCl 5M and purified by a liquid-liquid extraction with chloroform (100 ml). The organic layer was dried with anhydrous sodium sulphate and concentrated under reduced pressure, resulting in a brown oil that was used in the next step without further purification (procedure adapted from [93]).

5.2.2. Synthesis of 3,4-diethyl-1-methyl-9*H*-xanthen-9-one (4): cyclization of benzophenone intermediate 3

To a solution of NaOH (24 g, 0.6 mol) in MeOH (150 ml) and water (100 ml), the crude material containing 327,8 mg of (2-hydroxy-3,4-dimethoxy-6-methylphenyl) (methoxyphenyl) methanone (**3**) was added. The mixture was submitted to microwave (MW) irradiation, at open vessel conditions, for 8.5h, at 300 W of potency, 75 ºC of temperature and 1 bar of pressure. After completing the reaction and cooling at room temperature, the white solid formed was filtered under vacuum, washed with MeOH and dried. The retained solid was further submitted to a liquid-liquid extraction with dichloromethane (5 x 100 ml). The organic layer was dried with anhydrous sodium sulphate and concentrated under reduced pressure. The mother liquor resulting from the vacuum filtration was, again, filtered under vacuum, washed with MeOH and dried. The progression of reaction and the purification and identity steps were monitored by TLC using *n*-hexane/ethyl acetate in a proportion of 7:3, using a standard previously obtained as control. The experimental procedure was adapted from [94].

5.2.3. Synthesis of 1-(dibromomethyl)-3,4-dimethoxy-9*H*-xanthen-9-one (5)

A mixture of 3,4-diethyl-1-methyl-9*H*-xanthen-9-one (**4**, 2.50 g, 9.25 mmol), *N*-bromosuccinimide (3.29 g, 2.0 eq.) and benzoyl peroxide (0.67g, 0.3 eq.) in carbon

tetrachloride (80 ml) was refluxed for 2 h, at 80 ºC. The chromatographic system *n*-hexane/ethyl acetate (8:2), was used to observe the progression of the reaction, using the intermediate **4** and a standard previously obtained as control. Once completed the reaction, the suspension was cooled at 0°C and stirred 1-2 h. The precipitate was filtered under vacuum and washed with cold carbon tetrachloride. The mother liquor was evaporated and then purified by a flash column chromatography (silica gel, *n*-hexane/ethyl acetate in gradient). The obtained fractions containing the intermediate **5** were collected, concentrated under reduced pressure and submitted to a chromatography flash cartridge (silica gel, *n*-hexane/ethyl acetate in gradient). The pure fractions were gathered and concentrated under reduced pressure to obtain a white solid of 1-(dibromomethyl)-3,4-dimethoxy-9*H*-xanthen-9-one (**5**, 3.14 g, 80%). The purification steps and identity were monitored by TLC using *n*-hexane/ethyl acetate (8:2), using a standard previously obtained as control. The experimental procedure was adapted from [106].

5.2.4. Synthesis of 3,4-dimethoxy-9-oxo-9*H*-xanthene-1-carbaldehyde (LEM2)

A mixture of the dibromoxanthone derivative **5** (3.14 g, 7.34 mmol) and ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate (6,85 ml, 5 eq.) mixed with water was heated at 100°C and refluxed for 2.5 h. The mobile phase *n*-hexane/ethyl acetate (8:2) was used to observe the progression of reaction, using the intermediate **5** as control. The reaction was allowed to cool at room temperature and extracted with ethyl acetate (10 x 150 ml). The organic layer was dried with anhydrous sodium sulphate, concentrated under reduced pressure and the crude material was purified using a chromatography flash cartridge (silica gel, *n*-hexane/ethyl acetate in gradient). The pure fractions were gathered and concentrated under reduced pressure. The impure fractions, after being concentrated under reduced pressure, were crystallized from ethyl acetate/*n*-hexane (2:1). The obtained crystals were filtered under vacuum and washed with cold *n*-hexane. The purification steps were monitored by TLC using *n*-hexane/ethyl acetate (8:2), using the intermediate **5** as control. A light yellow solid was obtained, corresponding to 3,4-dimethoxy-9-oxo-9*H*-xanthene-1-carbaldehyde (**LEM2**, 1.33 g, 64%). The experimental procedure described here was adapted from [97].

5.3. Development of an HPLC method to quantify LEM2

The HPLC method was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system. The stationary phase used was a ACE C18 column with a particle size of 5 μm, with length of 25 cm and internal diameter of 4.6 mm (ACE). The samples were analyzed with the mobile phase water : methanol (25:75), using a isocratic elution, at a flux of 1.0 ml/min.

Stock standard solutions of **LEM2** (100 μg/ml) were prepared in water : methanol (25:75). Standard solutions were obtained by dilution of the freshly prepared stock standard solution with water : methanol (25:75) to give eight different concentrations over the range of interest (0,25 to 6,00 μg/ml). The peak areas of **LEM2** for each standard concentration were determined from the chromatogram. Data were fitted to least squares linear regression to obtain a calibration curve. Calibration curves were prepared and analyzed in triplicate on three different days.

5.4. Preliminary studies for the development of proliposomal solutions

Mannitol was sieved to collect the fraction with particle size < 125 μm. To prepare weight ratios of carrier: lipid (1:1), (2.5:1), (5:1) and (10:1), 0.2g, 0.5g, 1g and 2g of mannitol respectively, were placed in the round-bottomed flask, which was rotated under reduced pressure at 75 – 80 ºC, for about 30 minutes. The proliposomal formulation with no drug was prepared by a mixture of 171.02 mg of phosphatidylcholine and 29.0 mg of cholesterol (molar ratio 3:1) dissolved in 10 ml of EtOH. After the drying of mannitol, the temperature of the water bath was lowered and the ethanolic solution was added, in aliquots, to the round-bottomed flask, continuously rotated under reduced pressure until the solution had penetrated into the mannitol matrix. The solvent was completely evaporated. The experimental procedure was adapted from [49].

5.5. Production of proliposome

The lipid part of proliposomes was composed of a mixture of egg phospholipids with 80% of phosphatidylcholine and cholesterol. The carrier material used was mannitol.

5.5.1. Film deposition on carrier

Mannitol was sieved to collect the fraction with particle size < 125 μm, and 1.67 g of mannitol were placed in the round-bottomed flask, which was rotated under reduced pressure at 75 – 80 ºC, for about 30 minutes. The proliposomal formulation with no drug was prepared by a mixture of 142.52 mg of phosphatidylcholine and 24,17 mg of cholesterol (molar ratio 3:1) dissolved in 10 ml of EtOH. Proliposomes with 0.8%, 2% and 4% of **LEM2**, were prepared by adding respectively 1.33 mg, 3.33 mg and 6.67 mg of the compound to the ethanolic solution of lipids. After the drying of mannitol, the temperature of the water bath was lowered and the ethanolic solution was added, in aliquots, to the round-bottomed flask, continuously rotated under reduced pressure until the solution had penetrated into the mannitol matrix. The solvent was completely evaporated. The obtained proliposome powders were stored in a desiccator at room temperature. The experimental procedure was adapted from [49].

5.5.2. Freeze drying

Mannitol was sieved to collect the fraction with particle size < 125 μm. The proliposomal formulation with no drug was prepared by a mixture of 142.52 mg of phosphatidylcholine and 24,17 mg of cholesterol (molar ratio 3:1) and dissolved in 15 ml of EtOH. Proliposomes with 0.8%, 2% and 4% of **LEM2**, were prepared by adding respectively 1.33 mg, 3.33 mg and 6.67 mg of the compound to the ethanolic solution of lipids. The solution was subjected to ultrasonic agitation for 15 min. Mannitol was added to the solution and the volume of 100 ml was completed with distilled water. The solution was placed at a freeze dryer (VirTis Wizard 2.0, Advantage Plus, VirTis SP Scientific) and was initially frozen at -30 ºC, for 120min, at a vacuum pressure of 150 mTorr and with the condenser temperature -60 ºC. The primary

drying occurred at 20 ºC, for 600 min and the vaccum pressure 150 mTorr and a secondary drying stage was performed at 25 ºC, for 600 min and at the vacuum pressure of 100 mTorr.

5.5.3. Spray drying

A solution of 1% of mannitol in distilled water was sprayed through a 0.7 μm nozzle using a Nano Spray Dryer B-90 (Büchi, Switzerland) at an inlet temperature of 80 °C, an atomizing pressure of 33 mbar, and a feeding rate of 90 l/min. The outlet temperature was 37 - 38 °C. The product was separated and collected by the cyclone and then directed into the collecting chamber. The obtained mannitol particles were used as the carrier material to produce proliposomes by spray drying.

The proliposomal formulation with no drug was prepared by a mixture of 142.52 mg of phosphatidylcholine and 24,17 mg of cholesterol (molar ratio 3:1) and dissolved in 15 ml of EtOH. Proliposomes with 2% of **LEM2**, were prepared by adding 3.33 mg of the compound to the ethanolic solution of lipids. The solution was subjected to ultrasonic agitation for 15 min. Spray dried mannitol was added to the solution and the volume of 100 ml was completed with distilled water. The solution was subjected to ultrassonic agitation for 15 min in order to disaggregate mannitol particles. The solution was submitted to spray drying at a inlet temperature of 80 °C, an atomizing pressure of 33 mbar, and a feeding rate of 90 l/min. The outlet temperature was 37 - 38 °C. The obtained proliposome powders were stored in a desiccator at room temperature. The experimental procedure was adapted from [59].

5.6. Analysis of proliposome powders

5.6.1. Surface morphology of proliposomes

The surface morphology of proliposomes was analyzed by SEM. The SEM / EDS exam was performed using a High resolution (Schottky) Environmental Scanning Electron

Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M. Samples were coated with an Au/Pd thin film for 90 sec. and with a 15mA current, by sputtering, using the SPI Module Sputter Coater equipment. Each image contains a databar with the most important analysis conditions.

5.6.2. Thermal behaviour of proliposomes

Differential scanning calorimetry (DSC) analysis was carried out using a Netzsch DSC 200 F3 differential scanning calorimeter and Netzsch proteus thermal analysis software. DSC analyses were performed on the ingredients of the formulation and on proliposome powders on the day of production. In this way, the samples were weighed directly in aluminum pans and scanned between -20 °C and 240 °C at a heating rate of 10 °C/min.

5.7. Hydration of proliposomes

Proliposomes were hydrated to form liposomes. 60 mg of the proliposome powder were weighted and hydrated with 2 ml of ultrapure water and agitated manually for 1 min (adapted from [46]). The obtained solution was filtered through a 5 µm nitrocellulose membrane filter (Minisart, Germany) to remove possible lipid aggregates.

5.7.1. Surface morphology of liposomes

The surface morphology of liposomes was analyzed by CryoSEM. The SEM exam was performed using a High resolution Scanning Electron Microscope with X-Ray Microanalysis and CryoSEM experimental facilities: JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500. The specimen was rapidly cooled (plunging it into sub-cooled nitrogen – slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. The specimen was fractured, sublimated ('etched') for 90sec. at -90°C, and coated with Au/Pd by

sputtering for 30 sec and with a 12mA current. The sample was then transferred into the SEM chamber. The sample was studied at a temperature of -150°C. The conditions in which images were obtained are in the respective labels.

5.7.2. Particle size measurement

The particle size and polydispersity index was determined by dynamic light scattering (DLS), using a ZetaPALS, ZetaPotential Analyzer (Holtsville, NY, USA). Prior to the measurements, all samples were diluted (1:10) using purified water to yield a suitable scattering intensity, the average count rate indicates that the dilution applied to the formulations was appropriate. The measurements were always performed six times for each sample. The zetaPALS particle sizing software was used to analyze the obtained data.

5.7.3. Zeta potential measurement

Zeta potential of liposomes was measured using a ZetaPALS, ZetaPotential Analyzer (Holtsville, NY, USA). Prior to the measurements, all samples were diluted (1:10) using purified water to yield a suitable scattering intensity. The measurements were always performed six times for each sample. The PALS zeta potential analyzer software was used to analyze the obtained data.

5.7.4. Entrapment efficiency of liposomes

The amount of encapsulated drug within the nanoparticles was measured to calculate the encapsulation efficiency. Briefly, 220 mg of the freshly prepared proliposomal formulation was hydrated with 2 ml of ultrapure water and agitated manually for 1 min. The solution was filtered through a 5 μm nitrocellulose membrane filter (Minisart, Germany) to remove unencapsulated drug crystals. Then, 7 ml of methanol was added to 1 ml of filtered formulation and thoroughly mixed to extract the drug from the lipid matrix. The mixture was

then centrifuged at 5000 rpm for 15 min and the supernatant was diluted with methanol (1:1) and centrifuged at 5000 rpm for 5 min and the supernatant was filtered through a 0,45 μm PTFE syringe filter (Olimpeak by Teknokroma, Ireland). If needed, the supernatant was again diluted with the mixture of solvents water: methanol (25:75) to the HPLC assay calibration range. The amount of drug in the filtered supernatant was measured by HPLC. The amount of drug in the filtered formulation was then calculated considering the dilution factor. The experimental procedure was adapted from [107]. The entrapment efficiency was calculated by the following **equation 2**:

$$EE\ (\%) = \frac{Amount\ of\ drug\ in\ filtered\ formulation}{Total\ amount\ of\ drug}\ x\ 100 \qquad \textbf{Equation 2}$$

5.8. Stability studies

After the production of proliposomes, they were kept at a desiccator at room temperature. Their stability was studied 30 days after the storage. DSC data of proliposome powders was analyzed. After hydration of proliposomes, the particle size, zeta potential and entrapment efficiency of liposomes were also analyzed.

5.9. Statistical analysis

For the characterization of liposomes hydrated from proliposomes at the day of production results are shown as the mean ± standard deviation of 3 different batches of the same formulation. The t-test and the one-way analysis of variance (ANOVA) were performed to compare two or multiple groups, respectively. If the group by each time interaction was significantly different ($P < 0.05$), differences between groups were compared within a post hoc test (Tukey HSD). All statistical analyses were performed with the software IBM SPSS Statistics 23 (SPSS Inc., Chicago, USA).

CHAPTER 6
REFERENCES

CHAPTER 6 - REFERENCES

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CHAPTER 7
APPENDICES

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Appendix I - Characterization of liposomes formed by hydration of proliposomes with different carrier : lipid weight ratio, by freeze drying.

Table XIV - Characterization of liposomes formed by hydration of proliposomes with different carrier : lipid weight ratio, by freeze drying.

Carrier : lipid weigh ratio	Effective diameter (nm)	Polydispersity	Zeta potential (mV)
1 : 1	470.2 ± 32.7	0.328 ± 0.014	-32.03 ± 3.58
2.5 : 1	516.1 ± 4.0	0.324 ±0.010	-36.45 ± 3.31
5 : 1	529.0 ± 18.7	0.326 ± 0.009	-40.16 ± 11.40
10 : 1	443.6 ± 26.4	0.318 ± 0.030	-41.32 ± 0.74

Appendix II - Characterization of liposomes formed by hydration of proliposomes at the day of production

Table XV - Characterization of liposomes formed by hydration of film deposition on carrier proliposomes at the day of production.

Percentage of LEM2	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
No drug	546.5 ± 66.0	0.330 ± 0.03	-47.38 ± 8.19	
0.8 %	592.3 ± 42.6	0.344 ± 0.007	-38.31 ± 0.28	30.92 ± 8.80
2 %	614.6 ± 39.1	0.335 ± 0.007	-38.11 ± 0.06	51.92 ± 21.91
4 %	659.1 ± 152.4	0.353 ± 0.019	-38.38 ± 0.21	25.98 ± 23.81

Table XVI - Characterization of liposomes formed by hydration of freeze dried proliposomes at the day of production.

Percentage of LEM2	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
No drug	443.6 ± 26,8	0.318 ± 0.030	-41.84 ± 2.66	
0.8 %	450.6 ± 38.0	0.350 ± 0.015	-37.83 ± 1.49	24.23 ± 5.74
2 %	428.2 ± 18.2	0.337 ± 0.025	-38.63 ± 0.40	62.68 ± 31.55
4 %	410.8 ± 17.2	0.335 ± 0.025	-38.11 ± 2.62	4.72 ± 0.55

Table XVII - Characterization of liposomes formed by hydration of spray dried proliposomes at the day of production.

Percentage of LEM2	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
No drug	243.2 ± 34.0	0.272 ±0.024	-44.93 ± 2.84	
2%	272.2 ± 20.9	0.314 ± 0.004	-38.30 ± 0.19	47.54 ± 24.73

Appendix III – Characterization of liposomes formed by hydration of proliposomes at the day 30.

Table XVIII - Characterization of liposomes formed by hydration of film deposition on carrier proliposomes at the day 30.

Day	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
0	614.6 ± 39.1	0.335 ± 0.007	-38.11 ± 0.06	51.92 ± 21.91
30	625.9 ± 62.1	0.329 ± 0.019	-38.50 ± 0.16	10.47 ± 1.25

Table XIX - Characterization of liposomes formed by hydration of freeze dried proliposomes at the day 30.

Sample	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
0	428.23 ± 18.24	0.337 ± 0.025	-38.63 ± 0.40	62.68 ± 31.55
30	522.03 ± 33.57	0.327 ± 0.012	-38.21 ± 0.24	22.22 ± 24.13

Table XX - Characterization of liposomes formed by hydration of spray dried proliposomes at the day 30.

Day	Effective diameter (nm)	Polydispersity	Zeta potential (mV)	Entrapment efficiency (%)
0	272.2 ± 20.9	0.314 ± 0.004	-38.30 ± 0.19	47.54 ± 24.73
30	282.3 ± 64.0	0.297± 0.035	-38.35 ± 0.24	28.68 ±13.71